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CLONING, SEQUENCING AND EXPRESSION OF THE SECRETED ALKALINE PHOSPHATASE GENE FROM *LYSOBACTER ENZYMOGENES*

BY

SAMSON AU ©

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

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To my family and friends who have always been there when I needed them.

ABSTRACT

Lysobacter enzymogenes produces a secreted alkaline phosphatase, PhoA. This enzyme has a molecular weight of approximately 30 kDa and it is active in the presence of EDTA. The gene for the phosphatase, *phoA*, was located within a 4.4 kb *Eco*RI-*Bam*HI DNA fragment from a recombinant lambda phage library and its sequence was determined using the chain termination method. The phosphatase gene is 1620 bp in length and has a G+C content of 69.5%. It contains an open reading frame which encodes a 539 amino acid protein with four domains; a 29-residue signal sequence, a 119-residue amino-terminal propeptide, the approximately 281-residue mature phosphatase and an approximately 110-residue carboxy-terminal domain. The phosphatase precursor has been compared to other known precursor proteins, but the functions of the N- and C-terminal extensions remain to be determined.

Amino acid homology searches showed that the phosphatase is structurally related to mammalian iron-containing, purple acid phosphatases. The amino acid sequence, "LVGHDHNYQRY" located in the C-terminal half of the *L. enzymogenes* phosphatase is very similar to one present in the mammalian acid phosphatases, and it may be part of the iron-coordination/active site of the enzyme. Atomic absorption studies confirmed that a significant amount of iron is present in the enzyme, suggesting an iron to protein molar ratio of 1:1. No prokaryotic homolog of the phosphatase gene has yet been found.

The phosphatase gene was modified by PCR in order to provide useful cloning sites, a consensus ribosome binding site, and the ATG initiator codon, in order to improve expression in *Escherichia coli*. The amplified phosphatase gene was cloned into pUC118, tranformed into *E. coli* and the transformed cultures were induced with IPTG. The expressed protein was released from *E. coli* by cold-osmotic shock. The shock fluid and the culture supernatant contained active alkaline phosphatase which was inhibited by anti-

alkaline phosphatase antiserum but not by EDTA. The phosphatase found in the culture supernatant was probably the result of leakage from the periplasm. After partial purification of the shock fluid by gel filtration, there was a 3- to 6-fold increase in enzyme activity. The activation may be due to the removal of an inhibitory factor. Western transfers and immunoblots of shock fluid and partially purified preparations have detected proteins similar to the 30 kDa phosphatase and also proteins of higher molecular weight. This may indicate that some of the precursor was processed incompletely.

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List of Abbreviations

Amp	ampicillin
anti-AP	anti-alkaline phosphatase antibody
BSA	bovine serum albumin
bp	base pair(s)
Ci	Curie
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
EDDHA	ethylenediaminedi(*o*-hydroxyphenyl acetic acid)
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
GEP	general export pathway
g	force of gravity
IPTG	isopropyl-β-D-thiogalactoside
Kan	kanamycin
kb	1 000 base pairs
kDa	kilodalton
MOPS	4-morpholinepropanesulfonic acid
OAc	acetate ion (CH_3COO^-)
OVA	ovalbumin
*p*NPP	*p*-nitrophenylphosphate
PADAC	[7-(thienyl-2-acetamido)-3-[2-(4-*N*,*N*-dimethy-aminophenylazo)-pyridinium methyl]-3-cephem-4-carboxylic acid]
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol 8000
RBS	ribosome binding site
SDS-PAGE	sodium dodecy sulfate-polyacryamide gel electrophoresis
SRP	signal recognition particle
TE	Tris-EDTA buffer
TEA	Tris-EDTA-acetate buffer
TEB	Tris-EDTA-borate buffer
Tris	Tris(hydroxymethyl)aminomethane
XGAL	5-bromo-4-chloro-3-indolyl-galactoside
UV	ultraviolet (light)

CHAPTER 1

Introduction

1.1 *Lysobacter enzymogenes* and other Gliding Bacteria

Organisms belonging to the genus *Lysobacter* were originally isolated from soil, found to degrade chitin, and to move in an unconventional manner described as gliding (Whitaker, *et al.*, 1967). They also attracted considerable attention because they can lyse many organisms such as nematodes, fungi and other bacteria. Isolates which possessed some of the above characteristics were broadly assigned to the group *Myxobacterales*, the gliding bacteria.

Gliding bacteria are diverse in their morphology and physiology and are commonly found in freshwater, in soil and on surfaces such leaves and wood. They move in a gliding motion, perhaps by producing slime, the production of which is especially prevalent in nutrient-deficient environments. All are Gram-negative, rod-shaped organisms capable of chemoheterotrophic growth. None produce fruiting bodies, although microcysts are occasionally observed. Many of them also synthesize pigments (Reichenbach, 1992). They can decompose many macromolecules such as polysaccharides, lipids, proteins, nucleic acids and even whole cells. *Lysobacter*, due in part to the unusually high %G+C in its genomic DNA (Christensen and Cook, 1978), is thought to belong in a separate order from other gliding bacteria. All gliding organisms have been placed in a heterogeneous taxonomic group referred to as the Non-photosynthetic Non-fruiting Gliding Bacteria (Larkin, 1989) which include three major orders, the *Beggiatoales*, *Cytophagales* and

Lysobacterales. *Lysobacterales* contains only one family, *Lysobacteraceae*, and one genus, *Lysobacter*.

The genus *Lysobacter* contains four recognized species (Table 1-1) which are known for the many extracellular products they produce such as antibiotics, polysaccharides and enzymes. Of these, *L. enzymogenes* ATCC 29487, is the most extensively investigated. It has been shown to synthesize α-lytic protease and β-lytic protease (Olson, *et al.*, 1970; McLachlan and Shotton, 1971), myxosidin A and B (Clapin and Whitaker, 1978); nuclease (von Tigerstrom, 1980), ribonuclease (von Tigerstrom, 1981), two phosphatases (von Tigerstrom, 1984; von Tigerstrom and Stelmaschuk, 1985; 1986), two esterases (von Tigerstrom and Stelmaschuk, 1989) and β-lactamase (von Tigerstrom and Boras, 1990). In the cases of the esterases and the phosphatases, both membrane-associated and soluble forms occur.

Of these extracellular enzymes, the α-lytic protease has been studied in the greatest detail. The α-lytic protease is a serine protease with similar features to a porcine elastase (McLachlan and Shotton, 1971; Brayer, *et al.*, 1979). It is known to attack mucopeptides of bacterial cell walls (Kaplan, *et al.*, 1970; McLachlan and Shotton, 1971). This enzyme was shown by crystallographic and amino acid sequence comparisons with other proteases to be similar to the serine proteases A and B of *Streptomyces griseus* (Brayer, *et al.*, 1979; Henderson, *et al.*, 1987). More recently, the gene for the α-lytic protease has been cloned and sequenced. Like many extracellular proteases (Henderson, *et al.*, 1987; Ikemura, *et al.*, 1987; Bever and Iglewski, 1988; Ohta, *et al.*, 1991), the α-lytic protease is synthesized with a large N-terminal extension in addition to the signal sequence (Epstein and Wensink, 1987; Silen, *et al.*, 1988). The function of the propeptide is thought to be important in the activation of the protease (Baker, *et al.*, 1992; Creighton, 1992).

The remaining species all produce various extracellular enzymes and secondary metabolites, but they have not been investigated in much detail. *L. antibioticus* synthesizes a potent antibiotic called myxin (Peterson, *et al.*, 1966) which is used in veterinary

Table 1-1: Members of the genus *Lysobacter* *.

Organism	Products of Interest
Presently recognized species	
L. enzymogenes ATCC 29487 (UASM 495)	α-lytic protease, lipases, nucleases, phosphatases, glucanase, β-lactamase
L. enzymogenes AL-1 ATCC 27796	keratinase, elastase, glucanase, protease
L. antibioticus ATCC 29497	myxin, and various hydrolytic enzymes
L. brunescens ATCC 29482	amylase, and various hydrolytic enzymes
L. gummosus ATCC 29489	various hydrolytic enzymes
Proposed species	
L. lactamgenus	cephabacin
L. albus	lactivicin

* adapted from Larkin, 1989 and Reichenbach, 1992.

medicine. *L. brunescens* secretes an extracellular amylase (von Tigerstrom and Stelmaschuk, 1989). Enzyme studies using *L. gummosus* are difficult since it produces a heavy gum that makes liquid cultures extremely viscous. Recently, two new antibiotic-producing species of *Lysobacter* have been described which have similar characteristics to other *Lysobacter* sp. *L. lactamgenus*, which produces cephabacins, and *L. albus*, which produces lactivicin, have been placed in the genus but await further characterization (Ono, *et al.*, 1984; O'Sullivan, *et al.*, 1988; Nozaki, *et al.*, 1989; Reichenbach, 1992).

1.2 Exoproteins and Gram-negative Bacteria

Proteins make up over 50% of a cell's dry weight, and between one thousand and ten thousand different kinds of protein are produced by a cell (Alberts, *et al.*, 1989; Neidhardt, *et al.*, 1990). Most proteins have specific functions in the cytoplasm while the remainder leave the cytoplasm to be localized in subcellular compartments or secreted into the cell's environment. This localization and secretion requires the movement of relatively hydrophilic polypeptides through membranes which are generally impermeable to macromolecules. The mechanisms by which proteins are directed out of the cell have been extensively investigated in both eukaryotic and prokaryotic systems over the past twenty years and it has become evident that the mechanisms may differ between organisms and even between different exoproteins produced by the same organism. In eukaryotes, proteins cross the plasma membrane and the membranes of the ER, nuclei, mitochondria and chloroplasts (Alberts, *et al.*, 1989). In Gram-negative prokaryotes, proteins may remain in the cytoplasm, become embedded in or associated with the cytoplasmic membrane, and extracellular proteins may become localized in the periplasm, associate with the outer membrane or be released into the surrounding medium. Thus, like eukaryotes, extracellular proteins from Gram-negative bacteria may exist in different subcellular compartments. The majority of proteins that are secreted into the medium by bacteria are enzymes. Gram-positive organisms, because they do not possess an outer membrane, do not have a periplasmic space and usually secrete their extracellular proteins. Despite differences in cell wall structure, Gram-positive and Gram-negative organisms seem to export proteins by similar mechanisms (Pugsley, 1989).

It was once thought that Gram-negative organisms do not secrete proteins through the outer membrane, since the outer membrane posed a major barrier to the movement of macromolecules. At the time, it was difficult to determine whether the exoprotein reached the medium by true secretion or due to leakage of cell components through the outer membrane (Glenn, 1976; Poole and Hancock, 1983). Since then, there have been numerous reports showing that many Gram-negative bacteria are able to secrete proteins. Some of these proteins may have virulence functions while others are of value industrially and commercially. For instance, the production and secretion of elastase and exotoxin A by *Pseudomonas aeruginosa* are believed to be important factors in pathogenesis (Nicas and Iglewski, 1985). Table 1-2 lists a limited number of enzymes which are secreted by Gram-negative bacteria.

Much of the data on protein export have been obtained from studies with Gram-negative organisms such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas* spp. and *Erwinia* spp. and Gram-positive organisms such as *Bacillus* spp. and *Streptomyces* spp. As the information on protein export accumulated, it seemed that each protein was secreted by a different mechanism. A number of targeting signals have been identified for proteins destined to leave the cytoplasm (Pugsley, 1989). They are responsible for guiding the protein to its final extracellular location. As described in detail below, developments in the field of molecular biology have led to the elucidation of a general export pathway in prokaryotes and at least six distinct modes of secretion at the biochemical and/or genetic levels.

Table 1-2: Some secreted proteins from Gram-negative bacteria.

Protein	**Organism**	**Reference**
α-lytic protease	"myxobacter" 495	Gillespie and Cook, 1965
haemolysin	*Escherichia coli*	Felmlee, *et al.*, 1985
aerolysin	*Aeromonas hydrophila*	Howard and Buckley, 1983
pullulanase	*Klebsiella pneumoniae*	Michaelis, *et al.*, 1985
serine protease	*Serratia marcescens*	Yanagida, *et al.*, 1986
IgA protease	*Neisseria gonorrhoeae*	Pohlner, *et al.*, 1987
elastase	*Pseudomonas aeruginosa*	Kessler and Safrin, 1988
aqualysin I	*Thermus aquaticus*	Kwon, *et al.*, 1988
alkaline protease	*P. aeruginosa*	Guzzo, *et al.*, 1990
HA/protease	*Vibrio cholerae*	Häse and Finkelstein, 1991
amylase	*A. hydrophila*	Gobius and Pemberton, 1988
protease	*Myxococcus xanthus*	Coletta and Miller, 1986

Earlier publications on the subject of protein transport often use the term 'secretion' for the passage of polypeptides through the plasma membrane of prokaryotes and the term 'excretion' for their export to the medium. At present, the trend is to describe extracellular proteins or exoproteins as those that completely cross the cytoplasmic membrane and this process is referred to as translocation or export. Therefore, bacterial periplasmic and outer membrane proteins are translocated but not secreted. Secreted proteins are considered to be a special case of extracellular proteins that are released into the growth medium, whether or not they cross an outer membrane. In eukaryotes, secretory proteins are generally synthesized by ER-associated polysomes and translocated through the ER, not the cytoplasmic membrane. Use of the term 'excretion', which describes the removal of a substance not useful to the organism, is inappropriate since it has a connotation of waste. In fact, many of these products are important to the organism's physiology.

1.3 Translocation of Proteins

All cells are enveloped by a cytoplasmic membrane or plasma membrane. This membrane helps maintain the special internal conditions of the cell by forming an impermeable barrier and controlling the import and export of ions and macromolecules. The basic structure of all biological membranes is very similar. They are composed of four major kinds of phospholipids, a number of membrane proteins, and, in the outer membrane of Gram-negative bacteria, lipopolysaccharide. These components are assembled in a non-covalent fashion to form a lipid bilayer which is stabilized primarily by hydrophobic interactions. Membrane-bound integral proteins can be embedded within the bilayer *via* hydrophobic interactions with the long fatty acid chains. Peripheral membrane proteins are generally associated with the membrane *via* protein-protein interactions with integral proteins. These proteins perform specific functions in nutrient transport, energy generation, environment sensing, protein export and cell division (Alberts, *et al.*, 1989; Neidhardt *et al.*, 1990; Nikaido, 1992). With respect to protein transport, different organisms may have their own distinct systems for importing or exporting polypeptides (Hirst and Welch, 1988; Pugsley, 1989).

1.3.1 Characteristics and functions of signal sequences

Unlike cytoplasmic proteins, most extracellular and some integral cytoplasmic membrane proteins are synthesized with an N-terminal leader peptide or signal sequence, which functions as an export signal for directing the precursor to the membrane, a discovery which led to the proposal of the signal hypothesis (Blobel and Dobberstein, 1975a; 1975b; Emr, *et al.*, 1980). Deletions of the leader region of precursors resulted in intracellular accumulation of the protein (Kadonaga, *et al.*, 1984) which demonstrated its importance in translocation. Furthermore, there was evidence which indicated that signal sequences may inhibit the folding of preproteins into stable structures (Liu, *et al.*, 1989). Comparisons of the known leader peptides do not show any consensus amino acid sequences, nor any significant homology at the nucleic acid level, although all appear to have similar features and function (Randall and Hardy, 1989). The typical signal sequence contains between twenty and thirty amino acids, although some with as few as fourteen or as many as sixty amino acids have been found. The leader can be subdivided into three regions. There are usually one to three positively charged residues at the N-terminal end, which are thought to interact with the negatively-charged inner surface of the cytoplasmic membrane (Inouye, *et al.*, 1982; Boyd and Beckwith, 1990). This is followed by a core of nine to twenty hydrophobic amino acids which are capable of spanning the cytoplasmic membrane. The C-terminal end of the signal sequence contains a region of three to five residues which specifies the recognition and cleavage site for the signal peptidase. To identify potential signal sequences, a weight matrix is used for pattern recognition. It examines the occurence of a particular type of amino acid at a given position relative to the potential cleavage site. The statistical analysis uses a moving window of up to 20 residues to calculate the probability of an unknown sequence to be a signal sequence (von Heijne, 1986). The (-3, -1) rule predicts that the amino acid at position -1 relative to the cleavage site must be small, and that the amino acid at position -3 cannot be large and polar, aromatic or charged (von Heijne, 1983). Using these parameters, this method is over 87% accurate.

In prokaryotes, non-lipoproteins and lipoproteins are cleaved by signal peptidases I and II, respectively (Wolfe, *et al.*, 1983; Tokunaga, *et al.*, 1984; Dalbey, 1991) and released into the periplasm. The nature of the cleavage site appears to be a distinct turn structure which agrees with the proposed loop model of protein translocation (Duffaud and Inouye, 1988). Intraspecies and interspecies exchange of leader peptides between different precursors usually results in translocation of the protein (Benson, *et al.*, 1985; Freudl, *et al.*, 1988; von Heijne, 1988) which suggests that leader peptides have a common function.

The signal sequence alone, although essential, is not sufficient to direct translocation of proteins. Unlike proOmpA (Freudl, *et al.*, 1987), additional information in the mature protein sequence appears to be required for the export of MalE in *E. coli* (Ito and Beckwith, 1981; Kadonaga, *et al.*, 1984). Furthermore, specific sequences in the C-terminal region may be necessary for proper localization of alkaline phosphatase (Gentschev, *et al.*, 1990) and TEM β-lactamase (Koshland and Botstein, 1980; Minsky, *et al.*, 1986). Deletions of this region from the *E. coli* phosphatase results in normal initiation and processing, but the protein remains associated with the membrane because it may not be able to fold into its proper conformation (Gentschev, *et al.*, 1990). Fusion of a signal peptide from MalB, LamB or PhoE to a normally cytoplasmic protein such as LacZ results in accumulation of the hybrid protein in the cytoplasm. Sequences within LacZ prevent it from being exported. These fusions prevent translocation of other normally exportable proteins, presumably by blocking the secretory machinery (Tommassen, *et al.*, 1985) such that no further translocation activity can occur. Some cytoplasmic proteins, however, can be translocated provided that they are in export-compatible form (MacIntyre, *et al.*, 1987). Differences between cytoplasmic and exported proteins with respect to charge distribution, secondary or tertiary structure contribute to a phenomenon known as export incompatibility (Ito, *et al.*, 1981). MacIntyre and Henning (1990) have determined that mature sequences of secretory proteins do not contain positive translocation signals. Translocation of a precursor depends on several factors including the nature of the N-terminus, the amount of secondary and tertiary structure and the presence or absence of large hydrophobic sequences.

1.3.2 Co- and post-translational translocation

The modes by which proteins are directed through membranes were first understood in eukaryotes and can be broadly divided into two categories, co-translational and post-translational translocation (Randall, 1983). However, the functional distinction between the two is becoming less important (Zimmerman and Meyer, 1986). Transcription of DNA to produce mRNA occurs in the nucleus of eukaryotes. The mRNA is processed to a mature form and subsequently transported to the cytoplasm for translation. Co-translational translocation is the process by which proteins are synthesized with an N-terminal signal sequence and transported simultaneously across a membrane. Almost all secretory proteins are synthesized on ribosomes bound to the ER and translocated into the ER lumen (Rapoport, 1990). During translation, the signal sequence is the first segment of the precursor to emerge from the ribosome. The SRP, a complex ribonucleoprotein, recognizes and binds to the signal sequence (Seigel and Walter, 1988; Bernstein, *et al.*, 1989). The 54 kDa subunit of the SRP associates with the signal sequence (Lütcke, *et al.*, 1992) while the 7SL RNA interacts with the ribosomal RNA (Seigel and Walter, 1988; Rapoport, 1990). Elongation is temporarily arrested (Walter and Blobel, 1981) so that the protein is unable to fold into an export-incompatible conformation (Munro, 1991). The

SRP/precursor complex binds to a specific receptor on the membrane, called the docking protein (Gilmore, *et al.*, 1982), where translation continues and the protein is thought to traverse an as yet undiscovered tunnel in the membrane (Hartmann, *et al.*, 1989). The signal sequence is subsequently cleaved by a peptidase (Evans, *et al.*, 1986) and the protein is released into the ER lumen where modifications such as N-glycosylation, disulphide bridge formation and oligomerization occur.

SRP-like factors have been discovered in *E. coli* and yeast. The *ftsY*, *ffh* and the *ffs* genes encode essential products homologous to the docking protein, SRP54 and 7SL RNA in *E. coli*, respectively (Poritz, *et al.*, 1990; Ribes, *et al.*, 1990). *Saccharomyces cereviseae* has a 7SL RNA homolog called scR1 (Hann and Walter, 1991; Munro, 1991). These subunits are interchangeable and chimeric SRPs are functional (Römisch, *et al.*, 1989; Bernstein, *et al.*, 1989). The SRP-dependent translocation pathway appears to have been conserved through evolution and was probably the first translocation system to arise (Rapoport, 1991).

In post-translational translocation, nuclear-encoded, organelle-specific polypeptides are initially synthesized on free ribosomes in the cytoplasm and released as soluble entities (von Heijne, 1988; Hartl, *et al.*, 1989). The respective precursors are transported to the nuclear, mitochondrial or chloroplast membranes where they are translocated by membrane-specific machinery according to specific targeting signals encoded within the primary amino acid sequence. Additional factors called molecular chaperones, in conjunction with ribonucleoside triphosphates, are required to prevent aggregation and premature folding in order to maintain the protein in a transport-competent state (MacIntyre and Henning, 1990; Ellis and van der Vies, 1991; Knittler and Haas, 1992). The folding of a prepeptide into stable tertiary conformations inhibits its translocation (Eilers and Schatz, 1986; Randall and Hardy, 1986).

Molecular chaperones are a heterogeneous group of proteins with diverse functions, which have in common, the ability to associate with immature or denatured proteins

(Laskey, *et al.*, 1978; Lecker, *et al.*, 1989; Landry and Gierasch, 1991). They are divided into different families according to their apparent functions and then subdivided based on amino acid sequence homologies (Ellis and Hemmingsen, 1989; Ellis and van der Vies, 1991). The precise mechanisms of chaperone action are still unknown, but two observations are clear. Chaperones seem to form stable complexes with proteins such as proOmpA or prePhoE, since these complexes can be isolated *in vitro* (Lecker, *et al.*, 1990). They also seem to be transiently associated with proteins such as SecA or RNase A but do not form complexes (Lecker, *et al.*, 1989; Rothman, 1989). Chaperone binding is most likely mediated by hydrophobic interactions to prevent aggregation, premature folding and internalization of apolar domains of newly synthesized polypeptides. Proteins, such as SecB, have been found to maintain an exportable precursor in a loosely folded or unfolded state in preparation for translocation (Lecker, *et al.*, 1990; Kumamoto, 1991). BiP and Hsp70 are required for protein import and folding in the lumen of the ER and the mitochondria, respectively (Murakami, *et al.*, 1988; Rose, *et al.*, 1989; Kang, *et al.*, 1990; Manning-Krieg, *et al.*, 1991; Knittler and Haas, 1992). Another family of chaperones, which includes the heat-shock proteins, are involved in the refolding and/or disposal of denatured polypeptides arising from environmental stresses (Liberek, *et al.*, 1988; Bochkareva, *et al.*, 1988; Ellis and Hemmingsen, 1989; Lindquist and Craig, 1988; LaRossa and van Dyk, 1991). LacZ fusion proteins, which are not normally translocated, can be exported in the presence of the GroEL chaperone protein (Phillips and Silhavy, 1990). Some chaperones perform dual functions in the translocation and the mediation of correct folding and/or oligomerization of preproteins. It is becoming increasingly apparent that many proteins require the aid of chaperones at some stage of their maturation. However, some enzymes like ribonuclease have been shown to fold spontaneously *in vitro* (Anfinsen, 1973), but these proteins are not common. The present model, called assisted self-assembly, suggests that chaperones act, not by providing steric information, but by stabilizing the interactive precursor domains to promote kinetic and thermodynamically favored conformations (Rothman, 1989; Ellis and van der Vies, 1991).

1.3.3 The general export pathway

Despite major organizational differences between prokaryotic and eukaryotic cells, the mechanisms of protein translocation are very similar. Most prokaryotic extracellular proteins are post-translationally translocated through the cytoplasmic membrane via the General Export Pathway (Pugsley, 1989; Wickner, *et al.*, 1991). Co-translational translocation also occurs but the differences appear to be mainly temporal (Randall, 1983; Zimmerman and Meyer, 1986). Prokaryotic translocation has been studied most extensively in *E. coli*, and it is likely that other organisms have protein export systems that share similar characteristics (Schatz and Beckwith, 1990).

Genetic and biochemical studies have identified at least six proteins encoded by the *sec* genes in *E. coli*, shown in Table 1-3, which recognize an exportable preprotein and translocate it across the cytoplasmic membrane (Randall, *et al.*, 1987; Wickner *et al.*, 1991). Although the eukaryotic and prokaryotic translocation machinery components are not structurally related, they seem to have analogous functions. The mature domains of a newly synthesized precursor are stabilized by a chaperone protein encoded by *secB*. SecB, a multimer consisting of four identical 155-residue polypeptides, maintains a translocation-competent state by helping to prevent tight folding, mis-association and aggregation of the preprotein (Kumamoto and Nault, 1989; Watanbe and Blobel, 1989), but does not act as an unfoldase (Lecker, *et al.*, 1990). SecB is not essential for translocation, since some of its functions can, presumably, be replaced by other chaperones such as GroEL and DnaK (Johnson, *et al.*, 1989; Kusukawa, *et al.*, 1989; Lecker, *et al.*, 1989; Phillips and Silhavy, 1990; Altman, *et al.*, 1991). Co-translationally transported proteins are not likely to have significant secondary structure and may not require the aid of SecB. SecB has been found

Table 1-3: Genes involved in the General Export Pathway of *E. coli* *.

Gene	Function of Product
secA	binds to precursors with signal peptides, ATPase activity
secB	molecular chaperone, stablizes unfolded conformation of many pre-proteins.
secD	integral membrane protein with periplasmic domain, functions late in translocation prior to peptidase cleavage.
secE (*prlG*)	integral membrane protein.
secF	integral membrane protein with periplasmic domain, functions late in translocation prior to peptidase cleavage.
secY (*prlA*)	signal peptide receptor and/or transport channel.

* from Wickner, *et al.*, 1991; Sugai and Wu, 1992.

to be especially important in the translocation of pre-maltose binding protein but not necessary for several other exported proteins (Benson, *et al.*, 1984; Kumamoto, 1991; Sugai and Wu, 1992).

SecB differs from other chaperones in that it can be bound by the peripheral membrane protein SecA (Hartl, *et al.*, 1990), the part of the translocation complex which has ATPase activity (Lill, *et al.*, 1989; 1990). The SecA protein acts as a receptor for the SecB protein/precursor complex and binds to the signal sequence and mature domains of the precursor (Wickner,*et al.*, 1991). The SecA/SecB/precursor complex associates with SecE, SecY and an unknown gene product which make up the remainder of the integral membrane translocator complex, or translocon (Hartl, *et al.*, 1990). SecA binds ATP and permits limited translocation of the leader peptide through the membrane, where it is cleaved by the signal peptidase, after which ATP hydrolysis releases the precursor from SecA. The binding and hydrolysis of ATP by SecA may occur several times during the translocation process (Schiebel, *et al.*, 1991). The proton motive force drives translocation while the precursor is not bound to SecA (Schiebel, *et al.*, 1991).

Whether the SecY/SecE complex mediates translocation directly or functions merely as a pore is not known (Sugai and Wu, 1992). PrlA or SecY mutants are suppressors of signal sequence mutations, which suggests that SecY interacts directly with the leader (Emr and Silhavy, 1982; Shultz, *et al.*, 1982). Purified SecA, SecE and SecY have been reconstituted in proteoliposomes and found to be sufficient and essential for translocation activity (Akimaru, *et al.*, 1991). Two other genes, *secD* and *secF*, encode proteins with large periplasmic domains and function in the late stages of the translocation process prior to signal peptide cleavage (Stader, *et al.*, 1989; Gardel, *et al.*, 1990; Sugai and Wu, 1992).

1.4 Protein Targeting Signals

Protein targeting determines how a translocated protein is sorted and where it is ultimately localized. Signal sequences are a type of cleavable targeting sequence encoded within the N-terminal primary amino acid sequence of translocated proteins. However, they do not necessarily specify the final location of a protein (Benson, *et al.*, 1984). The control of protein traffic in cells depends on other types of signals (von Heijne, 1988). Targeting signals may exist internally or in the C-terminal regions of a preprotein. In eukaryotes, plasma membrane proteins and some secretory proteins reach the cell surface in a constitutive manner described as 'bulk flow' (Pfeffer and Rothman, 1987). The flow of proteins from the ER through the Golgi apparatus to the outside relies on the presence or absence of targeting information called 'signal patches', since all proteins at this stage are in a folded conformation. Mammalian resident ER proteins, which also move with the bulk flow, have a conserved C-terminal amino acid sequence 'KDEL' called a retention signal. This allows the resident protein to be recognized and retrieved by the ER retention system (Rothman, 1987; Pelham, 1990; Pidoux and Armstrong, 1992). In contrast, Golgi retention signals appear to exist in the transmembrane domains of proteins (Machamer, 1991). Proteins destined for the lysosome or the secretory vesicles are selectively removed from the bulk flow using a poorly characterized signal patch (Baranski, *et al.*, 1990). Lysosomal hydrolases bind specifically to mannose-6-phosphate receptors on the Golgi and are transported and fused to an endosome (Pfeffer and Rothman, 1987).

Most mitochondrial precursors possess N-terminal cleavable presequences which lack the hydrophobic domain and utilize hydroxylated and positively charged amino acids (Hurt, *et al.*, 1985; Keng, *et al.*, 1986; Hartl, *et al.*, 1989; Hartl and Neupert, 1990; Neupert, *et al.*, 1990). Mitochondrial import receptors (Pfaller, *et al.*, 1988) recognize these sequences and help the precursor insert into the mitochondrial outer membrane (Pfanner, *et al.*, 1991). Other internal targeting signals are responsible for intramitochondrial sorting (Hartl, *et al.*, 1987). Nuclear-encoded chloroplast precursors must be transported to the chloroplast and imported through the outer and inner membrane into the stromal space (Smeekens, *et al.*, 1990). The preprotein possesses an N-terminal transit peptide which contains sufficient information for transport into the chloroplast stroma (Keegstra, 1989) where it is cleaved by a peptidase (Robinson and Ellis, 1984). Some proteins are also further transported into the thylakoid lumen mediated by a signal peptide-like targeting signal which is processed by a thylakoidal peptidase (Smeekens and Weisbeek, 1989). Analogous findings are seen with nuclear protein import (Hall, *et al.*, 1984; Kalderon, *et al.*, 1984).

Inner membrane proteins of Gram-negative organisms which have a cleavable signal can be bound to the membrane in two ways. Their N-termini may be acylated by fatty acids to act as an anchor, or they may possess hydrophobic regions within the mature sequence which can act as a stop transfer signal and trap the protein within the membrane (Yost, *et al.*, 1983; Coleman, *et al.*, 1985; Davis, *et al.*, 1985; Davis and Model, 1985). Many cytoplasmic membrane proteins have leader peptides which do not resemble a classical signal sequence and are not cleaved by the leader peptidase. The relatively hydrophobic proteins are proposed to insert into the membrane by a mechanism known as the membrane trigger hypothesis. The N-terminal leader permits the emerging polypeptide to remain soluble. The lipid bilayer somehow triggers the folding of the protein such that it enters and spans the membrane (Wickner, 1979). The signal peptidase is an example of such a protein (Wolfe and Wickner, 1984).

The C-terminal regions of some exported proteins are essential for complete translocation. In some cases, the translocation process is initiated, but the proteins seem to remain associated with the membrane. *E. coli* alkaline phosphatase and β-lactamase are not released into the periplasm when about 25 amino acids of the C-terminus are deleted (Koshland and Botstein, 1980; Gentschev, *et al.*, 1990). However, maltose-binding protein is not affected by similar deletions (Ito and Beckwith, 1981). Outer membrane proteins are translocated across the cytoplasmic membrane like other extracellular proteins, but insert into the outer membrane after a major conformational change (Model and Russel, 1990). *E. coli* spheroplasts can secrete OmpF into the medium as a soluble monomer. In the presence of lipids, especially lipopolysaccharides, they assemble into mature porins as trimers (Sen and Hikaido, 1990). LamB has been found to require, in addition to the signal sequence, amino acid residues 27-39 of the mature protein for its export from the cytoplasm and residues 39-49 for localization into the outer membrane (Benson, *et al.*, 1984).

1.5 Secretion Mechanisms of Gram-negative Bacteria

For a protein to be secreted into the medium by a Gram-negative bacterium, it must traverse both the cytoplasmic and the outer membranes. An examination of different protein secretion mechanisms has suggested that each protein or group of proteins uses a different pathway (Lory, 1992), which depends on various targeting signals that exist on the polypeptide. At one time, it had been postulated that a protein could cross both membranes simultaneously through so-called zones of adhesion (Bayer, *et al.*, 1982), but this idea has since fallen into disfavor (Kellenberger, 1990). Alternatively, proteins could cross the cytoplasmic membrane first via the general export pathway and subsequently traverse the outer membrane with the aid of other factors. There are presently at least six recognizable types of secretion mechanisms. They can be divided into mechanisms which initally utilize the general export pathway and those that do not.

1.5.1 General export pathway-dependent secretion mechanisms

Polypeptides in this category are transported to the cell surface and the extracellular milieu in two distinct steps. The general export pathway first translocates precursors through the cytoplasmic membrane into the periplasm in a signal sequence-dependent manner. Subsequently, the protein is secreted using one of three presently recognized mechanisms which require the aid of poorly defined accessory factors.

The secretion of IgA protease from *Neisseria gonorrhoeae* (Pohlner, *et al.*, 1987) and the serine protease from *Serratia marcescens* (Yanagida, *et al.*, 1986) are examples of one type of GEP-dependent secretion mechanism. These two enzymes are synthesized as prepro-enzymes with a typical leader peptide which enables the precursor to reach the periplasm via the SecA/SecY translocon. The C-terminal domains of the two precursors are very hydrophobic and are approximately 60-70 kDa in size. These domains are believed to form a helper pore in the outer membrane through which the mature portion of the precursor can traverse. IgA protease is then released auto-proteolytically in an active form. Small deletions within the C-terminal region is sufficient to block secretion of the IgA protease (Pohlner, *et al.*, 1987). *E. coli* cells that contain the *iga* gene are able to produce and secrete the protein (Halter, *et al.*, 1984). Therefore, no other factors appear to be required for secretion of the IgA protease.

Secretion of cholera toxin (Hirst *et al.*, 1984b) by *V. cholerae* is also GEP-dependent. Enterotoxin of *E. coli* (Palva, *et al.*, 1984), although it is structurally and functionally related to cholera toxin, is not secreted. Each toxin is composed of two subunits, A and B, which are translocated separately into the periplasm where they oligomerize (Hirst and Holmgren, 1987a). One A subunit associates with five B subunits to form the mature holotoxin. The B subunit contains the secretory information (Hirst and Holmgren, 1987b). Whereas enterotoxin remains periplasmic (Hirst, *et al.*, 1984a), cholera toxin is subsequently secreted to the medium mediated by an outer membrane protein complex only known as a toxin secretory apparatus (Hirst, *et al.*, 1984b). Since the toxin has already attained its final three-dimensional conformation, the toxin secretory apparatus probably recognizes a secretory signal similar to a signal patch. A *Vibrio* mutant has been isolated which is defective in the secretion of *E. coli* enterotoxin B subunit and its own extracellular proteins. The nature of the mutation is believed to affect a common step in the secretion of toxins and other extracellular proteins (Leese and Hirst, 1992).

A third general export pathway-dependent secretion mechanism, called the general secretion pathway, is represented by the pullulanase of *K. pneumoniae* (Pugsley, *et al.*, 1991) and several exoenzymes of *P. aeruginosa* (Wretlind and Pavlovskis, 1984; Lazdunski, *et al.*, 1990; de Groot, *et al.*, 1991) *Erwinia* (He, *et al.*, 1991) and related species. The pullulanase (PulA), a maltose-inducible lipoprotein produced by a *Klebsiella* sp., is translocated in a signal sequence-dependent manner. The leader peptide is processed by the lipoprotein signal peptidase and the N-terminal cysteine is fatty acid-acylated. The enzyme is mobilized to the cell surface where it spontaneously enters the medium as pullulanase micelles. The second step requires products from a set of accessory secretion genes which, in the case of pullulanase, are adjacent to the *pulA* structural gene. Table 1-4 lists fourteen secretion-specific genes that have been identified in *Klebsiella*, *pulC* to *pulO* and *pulS* (Pugsley and Reyss, 1990; Pugsley, *et al.*, 1991) and the homologous *xcp* and *out* genes from *P. aeruginosa* (de Groot, *et al.*, 1991; Bally, *et al.*, 1992) and *Erwinia* sp. (Ji, *et al.*, 1989; He, *et al.*, 1991; Létoffé, *et al.*, 1991). The periplasmic intermediate of pullulanase is believed to fold into higher ordered conformations. As in the case for cholera toxin, the secretion apparatus must recognize a conformational signal or "patch" instead of a linear amino acid sequence. The signal patch appears to reside in the N-terminal portion of the polypeptide (Kornacker and Pugsley, 1990a). Hybrid proteins consisting of the N-terminal regions of PulA and alkaline phosphatase, a periplasmic protein, are completely secreted into the medium (Kornacker and Pugsley, 1990b).

The homology studies have suggested possible functions for some of the secretion factors. In *P. aeruginosa*, the *xcpA* gene (Bally, *et al.*, 1991) has been shown to be identical to *pilD*, which encodes the peptidase required for pilin precursor processing (Nunn, *et al.*, 1990). *pilD* mutants can neither produce pili nor secrete *xcp*-dependent

Table 1-4: Genes involved in the *sec*-dependent General Secretion Pathway of *K. pneumoniae* and the homologous genes from *P. aeruginosa* and *Erwinia* sp.*

K. pneumoniae	*P. aeruginosa*	*Erwinia*	function/location
pulC	n/a	n/a	inner membrane
pulD	n/a	n/a	outer membrane protein
pulE	*xcpR* (58%)	n/a	assembly of apparatus?
pulF	*xcpS* (51%)	n/a	assembly of apparatus?
pulG	*xcpT* (51%)	n/a	secretory apparatus?
pulH	*xcpU* (23%)	*outH* (75%)[a]	secretory apparatus?
pulI	*xcpV* (24%)	*outI* (62%)	secretory apparatus?
pulJ	*xcpW* (36%)	*outJ* (62%)	secretory apparatus?
pulK	*xcpX* (33%)	*outK* (55%)	
pulL	*xcpY* (31%)	n/a	
pulM	*xcpZ* (26%)	n/a	
pulM	n/a	n/a	inner membrane
pulO	*xcpA* (48%)	n/a	peptidase
pulS	n/a	n/a	lipoprotein

[a] Amino acid homologies of the *xcp* and the *out* gene products to the respective *pul* gene products are indicated.

Proteins transported by the above systems

PulA	(*K. pneumoniae*)	pullulanase
PhoA	(*P. aeruginosa*)	alkaline phosphatase
PelE	(*E. chrysanthemi*)	pectate lyase isozyme

* from He, *et al*, 1991; Lazdunski, *et al*, 1990; Pugsley, *et al*, 1991.

exoenzymes such as exotoxin A (Strom, *et al.*, 1991). XcpT to XcpW and PulG to PulJ have deduced amino acid sequences similar to the signal sequences of the prepilin subunit. PulO is homologous to XcpA and may have a similar physiological role in *K. pneumoniae* (Pugsley and Dupuy, 1992). XcpR and XcpS (PulE, PulF)resemble PilB and PilC. Since PilB and PilC are required for the assembly of pili, it is proposed that XcpR and XcpS are required for the assembly of XcpT-U proteins. These similarities have led to the proposal that the XcpA/PilD participates in the processing and assembly of the secretion apparatus and is not directly involved in protein transport (Bally, *et al.*, 1992). The secretion signal recognized by the Xcp system is poorly understood, but a possible signal has been localized in the central regions of exotoxin A (Hwang, *et al.*, 1987; Chaudhary, *et al.*, 1988).

Hybridization experiments using DNA representing the *xcpA* and *xcpR-Z* regions as probes found that these genes are present in a number of related Gram-negative bacteria such as *Aeromonas hydrophila* (Jiang and Howard, 1992), *Xanthomonas campestris* and three *Pseudomonas* spp. (de Groot, *et al.*, 1991). The data suggest that this mode of secretion has been conserved in these organisms and perhaps others (Filloux, *et al.*, 1990). However, the ability to secrete a particular exoenzyme may be specific to a given organism. For instance, the elastase of *P. aeruginosa* was not secreted by *P. putida* (de Groot, *et al.*, 1991) while the cellulases of *E. chrysanthemi* and *E. carotovora* were not secreted by the *out* secretion systems of heterologous hosts when the genes were exchanged (Py, *et al.*, 1991). Since these systems can distinguish between enzymes with similar functions from different species, it is likely that the homologies of the Pul, Xcp and Out proteins represent similarities of the basic components of the secretory machinery, but they do not specify the particular proteins to be secreted.

1.5.2 General export pathway-independent secretion mechanisms

Not all extracellular proteins require a classical N-terminal signal sequence in order to be translocated through the cytoplasmic membrane. One mechanism that does not is known as the specific secretion pathway. Proteins using this pathway are synthesized without an N-terminal signal sequence and are secreted into the medium without a periplasmic intermediate (Felmlee, *et al.*, 1985). Several bacterial toxins including haemolysin of *E. coli* (Holland, 1989; Holland, *et al.*, 1990), cytolysin (CyaA) of *Bordetella pertussis* (Glaser, *et al.*, 1988), leukotoxin (LktA) of *Pasteurella haemolytica* (Strathdee and Lo, 1989); and the enzymes alkaline protease (AprA) of *P. aeruginosa* (Guzzo, *et al.*, 1990; 1991a, 1991b), metalloprotease of *E. chrysanthemi* (Létoffé, *et al.*, 1990) and NodO from *Rhizobium* (Economou, *et al.*, 1990) use this mode of secretion. The gene *hlyA* from uropathogenic strains of *E. coli* encodes a protein that contains a C-terminal targeting sequence (Mackman, *et al.*, 1986; Holland, 1989). Extragenic products, whose genes are contiguous with *hlyA* (Gray, 1989), recognize a signal in the C-terminal domain of the mature protein and transport occurs in a one-step process through both membranes simultaneously (Gray, *et al.*, 1984; Koronakis, *et al.*, 1989). This would also suggest that translocation occurs post-translationally and therefore the protein may have to be unfolded prior to transport. The nature of this recognition site is not likely to be a signal patch since hybrid proteins containing the C-terminal signal can be translocated by the secretion apparatus (Gray, 1989). The secretion apparatus comprises three accessory gene products, listed in Table 1-5, which are thought to span the cell envelope (Morana, 1983; Mackman, *et al.*, 1986; Létoffé, *et al.*, 1990). They include two inner membrane proteins (HlyB, HlyD; PrtD, PrtE) and one outer membrane protein (TolC; PrtF) which are responsible and sufficient for the identification of the haemolysin or metalloprotease and their secretion in an ATP-dependent manner. The energy for the early stages of HlyA secretion is provided by the proton motive force (Koronakis, *et al.*, 1991). A fourth

Table 1-5: Extragenic factors required for the secretion of haemolysin and analogous proteins.

E.coli	*P.aeruginosa*	*E.chrysanthemi*	**function/location**
HlyA	AprA	PrtB	enzyme
HlyB	AprD	PrtD	inner membrane protein -ATPase
HlyD	AprE	PrtE	inner membrane protein
TolC	AprF	PrtF	outer membrane component?

* from Holland, *et al*, 1990; Guzzo, *et al*, 1991; Létoffé, *et al*, 1991.

protein, HlyC, activates the haemolysin and is not involved in secretion (Nicaud, *et al.*, 1985).

The export signal resides in the 38 C-terminal and 46 C-terminal amino acids of the metalloprotease and haemolysin, respectively (Nicaud, *et al.*, 1986; Delepelaire and Wandersman, 1990; Kenny, *et al.*, 1992). The haemolysin signal is sufficient for secretion of chimeric proteins to the medium (Mackman, *et al.*, 1987). When the haemolysin C-terminal signal is fused to alkaline phosphatase without an N-terminal leader, the normally periplasmic enzyme is secreted into the medium independent of the GEP (Gentschev, *et al.*, 1990). Trans-complementation studies have shown that the *apr* genes of *P. aeruginosa* can be substituted with the *prt* genes of *Erwinia* sp. to allow secretion of alkaline protease in *E. coli* (Guzzo, *et al.*, 1991a). Similarly, the metalloproteases of *S. marcescens* were secreted from cells which contain the *prt* genes (Létoffé and Wandersman, 1992). The secretion of chimeric proteins appeared to be limited by the size of the passenger molecule. Results suggest that either the large molecule folds into secretion-incompetent conformations or that the secretion channel is too small to accomodate the protein. Since preliminary data have shown that chaperones such as SecB, GroEL or GroES are not involved in the secretion process, it has been suggested that the secretion apparatus may have unfolding activity (Gray, 1989; Létoffé and Wandersman, 1992).

Yops, Yad and Ylp are plasmid encoded proteins produced by three *Yersinia* spp., *Yersinia enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*. They have neither the classical N-terminal signal sequence recognized by the Sec appartus nor the C-terminal haemolysin signal sequence (Michiels, *et al.*, 1990). The Yops secretion signal resides in the 48 N-terminal residues, and yop fusion proteins constructed with β-galactosidase, alkaline phosphatase or cholera toxin B subunit are secreted efficiently (Sory, *et al.*, 1990; Michiels and Cornelis, 1991). The lack of homology between signal regions of various yop proteins suggests that signal recognition by the transport apparatus is conformational (Michiels, *et al.*, 1991).

Pilins and colicins represent two other groups of secreted proteins. The *E. coli* prepilin has a classical leader peptide which allows it to be translocated (Dodd and Eisenstein, 1984). Additional gene products are responsible for assembly. *Pseudomonas* spp. have a unique type of pilus signal sequence that is composed of six N-terminal amino acids, MKAQKG. It targets the peptide to the cell membrane where it is cleaved; then the N-terminal phenylalanine is methylated (Sastry, *et al.*, 1985) and the pilin monomers are assembled. Studies with alkaline phosphatase fusions have shown that the initial 45 amino acids of prepilin are sufficient for export of the hybrid protein in both *P. aeruginosa* and *E. coli* (Strom and Lory, 1987).

The colicins do not possess any signal sequences. Their secretion is unusual in that they accumulate in the cytoplasm and are released by pseudolysis or localized permeability of the cell membrane (Jakes and Model, 1979; Cavard *et al.*, 1985).

1.6 Expression of Genes in Foreign Hosts

1.6.1 Features of expression vectors

The advent of modern recombinant DNA technology has permitted the rapid isolation, cloning and characterization of structural genes from a variety of eukaryotic and prokaryotic organisms. Frequently, sequence information from a well-characterized protein is used to isolate its gene. Analysis of long stretches of nucleotide sequences occasionally identifies cryptic genes that encode proteins with unknown functions. It is now becoming increasingly important to express a cloned gene *in vivo* in order to determine if it encodes the expected product, to ascertain the function of a protein or to produce industrial quantities of protein. Some genes can be expressed in a foreign host without any alterations to their nucleic acid sequences because the required features, such as the promotor, that are easily recognized by the host organism, are already present. In the cases where the gene is developmentally regulated, such as antibiotic production or sporulation genes, or if the gene is from an evolutionarily distant organism, specific transcription factors or regulatory sequences may be necessary for the efficient transcription of the gene.

Almost any vector, plasmid or phage, is capable of endowing a cell with the ability to express a recombinant protein if the conditions are optimal, especially if the regulatory regions of the gene are compatable in the new host. Very often though, they are not. A class of vectors called expression vectors has been developed to facilitate the expression of genes, by providing consensus regulatory information around the gene of interest. There are expresson vectors for many host systems including *E. coli*, *Streptomyces* spp., *Bacillus* spp., yeast and some mammalian cells (Winnacker, 1987). In the construction of an expression vector for *E. coli* hosts, a parent vector, such as pBR322 or pACYC184, is chosen to provide an antibiotic selection system and an origin of replication. Genetic elements obtained from other sources should include an inducible promotor and a Shine-Dalgarno sequence immediately preceding the multiple cloning site. The multiple cloning site is a short region which contains a series of unique restriction endonuclease cleavage sites. Promotors such as tac, λ P_L or T7 ϕ10 are regulated by the LacI repressor, the temperature sensitive λ cI857 repressor or the RNA polymerase from bacteriophage T7, respectively.

For optimal initiation of translation, a concensus *E. coli* RBS should be used (Shine and Dalgarno, 1975). Ideally, the region between the initiator codon and the Shine-Dalgarno sequence should be 8-10 nucleotides long and consist of primarily A and U residues (Hui, *et al.*, 1984; de Boer and Hui, 1990; Ringquist, *et al.*, 1992). The presence of secondary structure in the RBS may mask important elements such as the Shine-Dalgarno sequence and/or the initiator codon (Looman, *et al.*, 1986). A transcriptional terminator should follow the multiple cloning site to prevent run-on transcription of adjacent genes. The initiator codon AUG may be provided just before the multiple cloning site in cases where an N-terminal truncated or processed protein is to be expressed. GUG, UUG, AUA and AUU codons initiate translation in *E. coli* at a much lower efficiency (Gren, 1984; de Boer and Hui, 1990). To simplify the recovery and purification process, translocation of the protein to the periplasm or the medium can be accomplished by producing hybrid proteins with vectors containing appropriately positioned signal sequences from PhoE, MalE or OmpA (Ghrayeb, *et al.*, 1984; Duffaud, *et al.*, 1987; Stader and Silhavy, 1990) or C-terminal secretion signals from HlyA or PrtB (Gentschev, *et al.*, 1990; Létoffé and Wandersman, 1992).

With the development of PCR methodology (Saiki, *et al.*, 1988) and custom oligonucleotide synthesis, some of these features can be incorporated directly into any gene, provided that some sequence information is available. Alternatively, sequences flanking the foreign gene may be retained and used in the final construct to determine whether the original promotor and/or RBS function in the host cell.

1.6.2 Factors influencing gene expression

Although the above considerations are important for gene expression, other factors may affect the amount of protein produced. These include the origin of the gene, vector copy number, lethality of the protein, product localization, stability of the protein and efficiency of translation. For example, the expression of a eukaryotic gene in a prokaryotic host will likely not mimic the normal situation in the eukaryotic cell and result in modification and compartmentalization differences (Luzikov, 1988). The plasmid copy number is important since large amounts of vector may outnumber the available repressor molecules (Glick and Whitney, 1987). For instance, the LacI protein normally represses the genes making up the *lac* operon (Stryer, 1981). If the *lac* promotor-regulated gene encodes a potentially lethal protein, cell growth may not occur if there is leaky transcription. This can be minimized, for example, by providing a copy of the *lac*I^q gene, an over-producer of lac repressor, on the host chromosome or, preferably, on the vector to control the promotor. The elevated levels of LacI help to maintain a numerical balance between the repressor and the operator.

If the product is normally cytosolic, the accumulating protein may precipitate and form granules within the cell (Kane and Hartley, 1988). It may be possible to resolubilize the protein when denaturants such as urea are used. If solubilized, the active protein may not be recovered because of improper protein refolding. If the product is to be transported across the cytoplasmic membrane *via* the Sec pathway, large hydrophobic sequences which may act as stop-transfer signals should not be present on the precursor. Otherwise, the precursors will block the secretory machinery and prevent further protein translocation. A successfully translocated protein will be released into the periplasm, but may require accessory factors for its secretion (Pugsley, *et al.*, 1991). The presence of intracellular or extracellular proteases may result in premature degradation of the polypeptide, so the use of protease-deficient hosts is desirable (Luzikov, 1988)

Another important consideration for gene expression is the regulation of translation. It is affected by at least three variables; the half-life of the mRNA, the initiation of translation and peptide elongation. The stability of the messenger determines how long a protein will be translated. The half-life of an mRNA species is dependent on its initial concentration and its susceptibility to ribonucleases, which is in part determined by its sequence and structure (Belasco, *et al.*, 1985; Belasco and Higgins, 1988). The frequency of translation initiation determines how many protein molecules are synthesized at a given time from the same mRNA molecule. The presence of secondary and tertiary structure in the RBS may shield important elements such as the initiator codon and the Shine-Dalgarno sequence and prevent ribosomes from initiating translation (Looman, *et al.*, 1986). The codon following the initiator codon appears to affect translation efficiency, not by codon preference and levels of the tRNA, but by the alteration of binding of the mRNA to the 16S

RNA via the RBS (Looman, *et al.*, 1987). Rare codons are believed to be involved in the control of some developmentally regulated proteins such as antibiotic synthesis enzymes in *Streptomyces* spp. (Leskiw, *et al.*, 1991). The presence of rare codons could potentially slow or arrest elongation and significantly reduce yield due to lack of sufficient numbers of corresponding tRNAs to decode them (Pedersen, 1984; Bonekamp, *et al.*, 1989; Sorensen, *et al.*, 1989; Chen and Inouye, 1990). However, codon preference and tRNA availability have been shown to influence the rate of translation in some systems, but not in others (Holm, 1986; Folley and Yarus, 1989).

Sequences just upstream of the RBS of ATP-synthase mRNA seem to regulate the production of stoichiometric amounts of the various subunits endcoded by the *atp* operon (McCarthy, *et al.*, 1985). An interesting finding is that the second and/or subsequent genes in an operon do not necessarily need a Shine-Dalgarno sequence for reinitiation of translation. This is probably due to the short spacing between the genes and the increased local concentration of ribosomes that have just completed translation of the previous gene around the next initiator codon (Ryoji, *et al.*, 1981). This observation was useful in the development of a dicistronic expression system that involved positioning the gene of interest immediately downstream of a known gene that has its own promotor, RBS and initiator codon (Ito and Kurosawa, 1992).

The expression of genes in heterologous hosts involves numerous variables and complex interactions, any of which can mean the difference between overproducing and obtaining barely detectable amounts of protein. Expression experiments are largely empirical. Therefore, the growth of cells, the detection conditions and the purification procedures must be optimized for each protein.

1.7 Objectives

A number of extracellular hydrolytic enzymes produced by *L. enzymogenes* were identified and characterized in this department. The publication by von Tigerstrom and Boras (1990) cites the references regarding these enzymes. Among the exoenzymes, there are two phosphatases, a cell-associated enzyme and a secreted enzyme. The cell-associated phosphatase has a molecular weight of about 69 kDa, a pH optimum of 8.5 and it is strongly inhibited by metal ion chelators such as EDTA. The secreted phosphatase is relatively small in size of about 25 kDa and has a pH optimum of 7.5, but unlike the other alkaline phosphatases (Torriani, 1968; Petitclerc, *et al.*, 1970; Day and Ingram, 1973; Yeh and Trela, 1976; Kobori and Taga, 1980), it is monomeric and insensitive to EDTA. Since *L. enzymogenes* produces many secreted proteins, it was thought that phosphatase would be very suitable for the study of protein translocation and secretion. The secreted phosphatase was selected to be investigated first because it is small, readily purified from the medium and easily differentiated from other phosphatases.

So far, only two other genes from *L. enzymogenes* have been studied. The gene for the α-lytic protease was investigated in two laboratories (Epstein and Wensink, 1987; Silen, *et al.*, 1988). The α-lytic protease gene has been sequenced and expressed in *E. coli*. The protease is synthesized as a precursor with a signal sequence and a large N-terminal propeptide. The role for the propeptide in protein folding and enzyme activation has been studied (Silen and Agard, 1989; Silen, *et al.*, 1989; Baker, *et al.*, 1992; Creighton, 1992). The gene for the β-lactamase, encoding a periplasmic enzyme, was characterized in this laboratory (Boras, *et al.*, 1993). Expression experiments using *E. coli* demonstrated that most of the *L. enzymogenes* β-lactamase activity remained cell-associated, probably in the periplasm (Boras, *et al.*, 1993).

The purpose of my project was to isolate the gene encoding the secreted alkaline phosphatase from genomic DNA, to compare the amino acid sequence to previously characterized proteins and to synthesize the phosphatase in an unrelated organism. It is hoped that experiments to express the phosphatase in *E. coli* or other hosts may yield information about the nature of precursor processing, the targeting signals and other factors involved in its secretion from *L. enzymogenes*.

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CHAPTER 2

Material and Methods

2.1 Materials

2.1.1 Reagents, enzymes and supplies.

Restriction endonucleases, T4 DNA ligase, polynucleotide kinase, the Klenow fragment of *E. coli* DNA polymerase and T7 DNA polymerase were purchased from Bethesda Research Laboratories Inc., [Gaithersburg, MD], Boehringer Mannheim Biochemicals, Canada , [Laval, Québec], New England Biolabs, [Boston, MA] or Pharmacia LKB Biotechnology [Uppsala, Sweden]. Avian myeloblastosis virus reverse transcriptase and Sequenase™ were products of Promega [Madison, WI] and U.S. Biochemicals Corp. [Cleveland, OH], respectively. Taq DNA polymerase, agarose and low melting point agarose were obtained from Boehringer Mannheim Biochemicals. *p*NPP was a product from the Sigma Chemical Co., [St. Louis, MO] and PADAC and Zwittergent 3-14 were obtained from Calbiochem, [San Diego, CA]. Various types of filter paper and carboxy-methyl ion exchange resins were from Whatman Paper Ltd, [Clifton, NJ].

M13 universal primer, reverse primer and a probe based on the N-terminal sequence of the mature enzyme were synthesized by the Regional DNA Synthesis Laboratory at the University of Calgary, Calgary Alberta. Subsequent oligonucleotide probes, sequencing primers and random primers were synthesized in our Department using an Applied Biosystems DNA Synthesizer model 381A or 391, Applied Biosystems Inc. (Foster City, CA). A list of primers, their locations and functions are listed in Table 2-1.

[α-^{32}P]dATP and [γ-^{32}P]ATP were obtained from New England Nuclear [Boston, MA] or ICN Radiochemicals [Irvine, CA.]. ^{35}S-methionine and ^{125}I-protein A were purchased from ICN. Nitrocellulose (Hybond-C) and nylon membranes (Hybond-N) were from Amersham Corp. [Arlington Heights, IL]. HATF filter discs were from Millipore Corp. [Bedford, MA]. These materials were used according to instructions of the suppliers. Ampicillin, kanamycin, carboxypeptidase P and salmon testes DNA were products of the Sigma Chemical Co. and media components were obtained from Difco Laboratories [Detroit MI], BBL Microbiology Systems [Cockeysville, MD] or Scott Laboratories Inc. [Fiskeville, RI]. Autoradiography was performed using Kodak XAR5 X-ray film Eastman Kodak Co. [Rochester, NY] or NIF RX from Fuji [Japan]. Agarose and SDS-PAGE gels were photographed with Polaroid 665 or 667 black and white instant film. Chemicals of reagent grade were purchased from various commercial sources.

2.1.2 Bacterial strains, vectors and culture conditions

Lysobacter enzymogenes ATCC 29487 (UASM 495) was used as a source of alkaline phosphatase and genomic DNA. *Escherichia coli* strains LE392, JM83, MV1193 and SMR10 were used for the production of a recombinant lambda phage library (Sorge, 1988), for the isolation of pUC recombinants, for production of single-stranded plasmid DNA and production of λ packaging extract (Rosenberg, 1987), respectively. Subsequently, only *E. coli* MV1193 was used for isolation of recombinant plasmids. In expression experiments which require a heat or an IPTG inducible T7 RNA polymerase, *E. coli* K38/GP1-2 (Tabor and Richardson, 1985), or *E. coli* JM109 (DE3) (Studier and Moffatt, 1986) were utilized, respectively. Recombinant DNA fragments were subcloned into either pUC118 or pUC119, and M13K07 helper phage was used for generation of

Table 2-1: List of oligonucleotide probes and sequencing primers.

Name	Sequence
#1	GG(G/C)AACGT(G/C)GT(G/C)GT(G/C)GT(G/C)GC(G/C)GG(G/C)GC(G/C)GG(G/C)GGCGACATC -phosphatase probe from N-terminal amino acid sequence, 33-mer
#2	ATGAACCC(G/C)GACACCGC(G/C)GC(G/C)GC -probe generated from CNBr fragment #4, 23-mer
#3	CAGGGCACCTC(G/C)GACCT(G/C)ATCGT -probe from N-terminal a.a. sequence, position 790, 23-mer
#4	ACGAT(G/C)AGGTCGCTGGT -reverse probe (N-terminal), serine variation 1, position 812, 17-mer
#5	ACGAT(G/C)AGGTC(G/C)GAGGT -reverse probe (N-terminal), serine variation 2, position 812, 17-mer
SAU1	CCAGCTTGAGCTGCTT -reverse sequencing primer, position 538, 17-mer
SAU2	GCTACTTCGACTACTTC -forward sequencing primer, position 974, 17-mer
SAU3	CTACTCGGGCTACAGCC -forward sequencing primer, position 1202, 17-mer
SAU4	TGTAGCAGTTTTCCGGC -forward sequencing primer, position 185, 17-mer
SAU5	GCACCTTCGGCGTTGCTC -forward sequencing primer, position 1472, 18-mer, accidental T-insertion

SAU6	TGCCCTGTTGCAGGTGC	-reverse sequencing primer, position 1540, 17-mer, missing one T
SAU7	GTTCACTGGCTGTCTCC	-reverse sequencing primer, position 294, 17-mer
SAU8	AATGTGTCGTCCGCCGC	-forward sequencing primer, position 1683, 17-mer
SAU9	TCGCCAACGACGGCGTG	-forward sequencing primer, position 1929, 17-mer
SAU10	CGCTGACCTGCAGCTTG	-reverse sequencing primer, position 1744, 17-mer
SAU11	GCATCAAGGAATCGGTC	-reverse sequencing primer, position 2213, 17-mer
SAU16 (T7BS)	AATACGACTCACTATAG	-T7-promotor sequencing primer, 17-mer
SAU21	CTGGGCGGCGTGCGCGGGCGCGAG	-reverse sequencing primer for double-stranded sequencing, position 381, 24-mer
SAU 28	CTTGAAGCCCACGCGTAGCCACTT	-reverse sequencing primer for Taq polymerase sequencing, position 525, 24-mer
SAU32	GCTGTTGTATTCGCTCA	-reverse sequencing primer, position 888, 17-mer

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* position numbers indicate the first nucleotide of the primer according to the sequence shown in Figure 3-2.

single-stranded phagemids (Messing, 1983; Vieira and Messing, 1987). PCR amplified DNAs (Saiki, *et al.*, 1988) used for gene expression were cloned into pBluescriptII KS$^+$, pT7-3, pT7-7 (Tabor and Richardson, 1985), pKK223-3 (Pharmacia), pTTQ18 (Stark, 1987), or pUC118 and used to transform *E. coli* MV1193. Recombinant T7 vectors were used to transform *E. coli* K38/GP1-2 or JM109 (DE3) for *in vivo* expression and labelling experiments.

Components making up the various media and buffers are shown in Table 2-2. Cultures of *L. enzymogenes* were maintained on SM agar. Inoculum was grown overnight in 0.8% tryptone or 0.8% soy peptone broth with shaking at room temperature. For enzyme production, 0.8% tryptone or 0.8% soy peptone broth was inoculated with a 2% (v/v) of the overnight culture. Cells were grown at room temperature for 18 h. For DNA isolation, cells were grown in 3% trypticase soy broth for 18 hr. *E. coli* SMR10 was grown on 2 x YT agar at 30°C and stored at 4°C. *E. coli* LE392, JM83 and MV1193 cultures were maintained on 2 x YT agar and grown in 2 x YT broth at 37°C for preparation of competent cells (Morrison, 1979). Alternatively, cultures were grown in LB broth and stored in TSS broth to prepare competent cells according to the method of Chung, *et al.* (1989). Transformed cells were grown on 2 x YT agar containing 150 μg/mL of Amp at 37°C. For the selection of recombinant plasmids, 50 μL of 2% XGAL in dimethylformamide and 10 μL of 100 mM IPTG were added to aliquots of transformation reactions prior to plating. Cultures for quick plasmid preparations were grown in 2 x YT broth plus 150 μg/mL of Amp. Cultures for expression experiments were grown in modified LB plus 150 μg/μL of Amp and, when required, induced with IPTG at a final concentration of 0.5 mM.

Table 2-2: List of media and solutions.

medium/buffer	components
2 x YT	1.6% tryptone, 1% yeast extract, 0.5% NaCl, +/- 1.5% agar
Denhardt's (50 x)	1% each of Ficoll, polyvinylpyrollidone, and BSA in H_2O
enzyme dil'n buffer	10 mM Tris/HCl, 50 mM NaCl, 1.5 mM $MgCl_2$
hybridization sol'n	6 x SSPE, 5 x Denhardt's, 0.5% SDS
Luria-Bertani (LB)	1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, +/- 1.5% agar
LBK	1% tryptone, 0.5% yeast extract, 1% NaCl, 4 mM NaOH
lysis buffer	0.5% SDS, 1 mM EDTA, 10 mM Tris/HCl, pH 7.5, 4 M guanidine isothiocyanate
modified LB	LB plus 50 mM MOPS buffer, pH 7.2
phage dil'n buffer	0.1 M NaCl, 20 mM Tris/HCl, pH 7.4, 10 mM $MgSO_4$
PBS	140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 35 mM K_2HPO_4, pH 7.0 with HCl
SM agar	1% skim milk, 0.2% tryptone, 1.5% agar
SM buffer	0.1 M NaCl, 10 mM MgSO4, 20 mM Tris/HCl, pH 7.5, 0.1% gelatin
SSPE buffer	0.15 M NaCl, 20 mM $NaPO_4$, pH 7.0, 2 mM EDTA
TE buffer	10 mM Tris/HCl, pH 8.0, 1 mM EDTA
TEA buffer	20 mM Tris base, 50 mM NaOAc, 2 mM EDTA
TEB buffer	60 mM Tris base, 60 mM boric acid, 1.2 mM EDTA
TSP buffer	30 mM Tris/HCl, pH 7.9, 7.5 mM spermidine, 7.5 mM putrescine
TSS	LB plus 10% PEG, 5% DMSO, 50 mM $MgCl_2$
Western buffers:	
tank buffer	5 mM Tris base, 20 mM glycine, 20% methanol
Solution I	15 mM NaCl, 5 mM EDTA, 5 mM Tris base, 0.25% gelatin, 0.05% p40 Nonidet
Solution II	1 M NaCl, 5 mM EDTA, 5 mM Tris/HCl, pH 7.5, 0.25% gelatin, 0.4% N-lauroyl sarcosine

2.2 Purification of alkaline phosphatases from *L. enzymogenes*

2.2.1 Assay for phosphatase activity

Activity of the secreted phosphatase was determined by pipetting 50 µL of enzyme sample into 450 µL of 1.2 mM *p*NPP, 2 mM EDTA, 0.6 M Tris/HCl, pH 7.5. After incubation at 37°C for 10 min, reactions were terminated by addition of 1 mL of 0.1 M K_2HPO_4, 0.15 M KOH and the absorbance was measured at 400 nm. Samples of cell-associated phosphatase were assayed with 4 mM *p*NPP, 0.6 M Tris/HCl, pH 8.5. When required, enzyme samples were diluted appropriately with enzyme dilution buffer. One unit of phosphatase activity is the amount of enzyme which hydrolyzes 1 µmol of *p*-nitrophenyl phosphate per min at 37°C. An extinction coefficient of 18 300 for *p*-nitrophenol was used to calculate enzyme activity.

The effect of metal chelators on the secreted phosphatase was examined by pre-incubation of the enzyme with 1 to 5 mM of EDTA, phenanthroline, dipyridyl or EDDHA at 37°C for 60 min prior to the standard assay.

2.2.2 Purification of the secreted alkaline phosphatase

The phosphatase was purified from *L. enzymogenes* ATCC 29487 according to the published method (von Tigerstrom, 1984) with a few minor modifications. Unless otherwise specified, all procedures were carried out at temperatures between 0° and 4°C. Ten 200 mL cultures were centrifuged at 15 000 x g for 10 min. The supernatant was retained and treated with 12 g QAE Sephadex for 30 min. The mixture was filtered through a sintered glass funnel to remove the Sephadex and the filtrate was recovered. An equal volume of cold deionized water was added to the filtrate. Approximately 500 g wet CM-52 cellulose, in the NH_4^+ form, was mixed with the filtrate, and the pH was adjusted to 5.0

with glacial acetic acid. After 30 min, the mixture was poured into a 5 cm diameter column and washed with 1 L of 10 mM NH_4OAc, pH 5.0. This was followed by elution with 1 L of 0.5 M NH_4OAc, pH 5.0. Fractions of 10-12 mL were collected. The active fractions were pooled and dialyzed against 2 L of 10 mM NH_4OAc, pH 5.0, for 2 x 4 h. This pool was designated the CM-52 enzyme concentrate. For larger scale enzyme purifications, preparations from 2 or 3 separate CM-52 concentrates were combined and dialyzed before continuing. The dialyzed CM-52 concentrate was applied to 250 mL of a freshly equilibrated CM-52 cellulose column. The column was washed with 250 mL of 10 mM NH_4OAc, pH 5.0 and the phosphatase was eluted with a gradient consisting of 600 mL of 10 mM NH_4OAc, pH 5.0 and 600 mL of 0.5 M NH_4OAc, pH 5.0, collecting fractions of 8-10 mL, and active fractions were pooled, dialyzed and lyophilized. The enzyme was redissolved in 5.0 mL of cold deionized water and sucrose was added to 10% (w/v). The enzyme solution was applied to a 2.5 cm x 40 cm (206 mL) Sephadex G-75 Superfine column equilibrated with 200 mM NaCl, 10 mM Tris/HCl, pH 7.5 and eluted with the same buffer. Fractions of 2.5 mL were collected and active fractions were pooled, dialyzed and lyophilized.

2.2.3 Preparation of the cell-associated phosphatase

The cell-associated phosphatase was prepared by a procedure based on the method of von Tigerstrom and Stelmaschuk (1986). *L. enzymogenes* was grown in 200 mL of 0.8% soy peptone for about 20 h and the cells were harvested by centrifugation. The pellet was washed in 20 mL of 2 mM $MgCl_2$ 10 mM Tris/HCl, pH 7.5 and resuspended in 20 mL of the same volume of buffer. The cells were disrupted using a French pressure cell and the mixture was centrifuged at 2 000 x g for 10 min. The supernatant was recentrifuged at 48 000 x g for 30 min. The pellet was resuspended in 5 mL of buffer and 1 mL of the suspension was solubilized with 0.2% Zwittergent 3-14 and incubated at 37°C for 15 min and then centrifuged for at 27 000 x g 15 min. The supernatant containing the enzyme was retained and assayed for phosphatase activity.

2.2.4 SDS-polyacrylamide gel electrophoresis

Samples of up to 20 µg of purified phosphatase or other protein was dissolved in up to 50 µL of gel loading dye (10 mM Tris/HCl, pH 8.0, 1.0% SDS, 10% glycerol or sucrose, Bromophenol Blue dye, +/- 0.1% β-mercaptoethanol) and heated to 95°C for 10 min. The proteins were separated on a 10% SDS-polyacrylamide gel using constant voltage (Maizel, 1971), except for CNBr fragments which were separated on a 12% gel. Voltages were set at 100 V until the samples entered the stacking gel, 200 V until the samples entered the separating gel and 300 V until the Bromphenol Blue tracking dye reached the bottom of the gel. After electrophoresis, the gel was fixed in 40% methanol, 7% HOAc overnight, stained with Coomassie Blue (0.5% in 25% isopropyl alcohol , 10% HOAc) for 2 h, destained with 10% isopropyl alcohol , 10% HOAc and stored in 10% HOAc (Fairbanks, *et al.*, 1971).

2.2.5 Production of anti-alkaline phosphatase antibody

Lyophilized enzyme was dissolved in sterile PBS to approximately 2.5 mg/mL and an aliquot was diluted 10-fold. Aliquots containing 25 µg of protein were spread onto each of two 4 cm^2 squares of Hybond-C nitrocellulose filters and allowed to dry overnight. The filters were soaked in 3 mL of cold PBS and cut into small pieces. The mixture was sonicated until it became powder-like. Two rabbits, a cross between the Flemish Giant and the French Lop Eared rabbits (University of Alberta Biosciences Animal Service), were each treated with two 0.25 mL subscapular injections and two 0.5 mL subdermal injections of the mixture for a total of approximately 50 µg of protein per rabbit. However, for subsequent injections, the same amount of purified phosphatase was immobilized onto two 4 mm x 35 mm nitrocellulose strips as above and one strip was implanted subdermally in each animal. The rabbits were bled after two weeks to obtain serum samples and the animals were injected with antigen again one week later. Blood samples were allowed to coagulate and the serum was decanted. The remaining particulate matter in the serum was removed by centrifugation at 12 000 x g for 15 min. Aliquots were stored in 300 μL portions at -20°C. This process was repeated five times over a period of approximately four months. Since the immune response was poor with this method, the rabbits were injected with 200 μg of total protein per rabbit in combination with Freund's complete adjuvant for the initial injections. The purified phosphatase was dissolved to 1.5 mL of PBS and homogenized with an equal volume of adjuvant. The injections were divided evenly between the two rabbits and administered subscapularly and subdermally as described before. Freund's incomplete adjuvant was used for two subsequent injections at three-week intervals.

The effect of the antiserum on phosphatase activity was determined by pre-incubating up to 0.5 μg of purified enzyme or 0.08 units of an enzyme sample with a known dilution of antiserum at 37°C for 60 min prior to the standard phosphatase assay. Enzyme and antibody were diluted with enzyme dilution buffer plus 10% normal serum. Samples were assayed before and after centrifugation to determine if any precipitation had occurred. Double antibody inhibition experiments (Midgely and Hepburn, 1980) were also performed. Up to 0.1 units of phosphatase in 50 μL of buffer was mixed with 12.5 μL of diluted antiserum for 45 min at 37°C. Fifty μL of undiluted goat anti-rabbit antiserum was added and incubation continued for another 30 min, and the mixture was chilled on ice for 1 h. The samples were centrifuged and assayed for phosphatase activity. A similar procedure using preparations of the cell-associated phosphatase was carried out as a control.

2.3 Amino acid sequence analysis of the secreted phosphatase

2.3.1 Cyanogen bromide cleavage

The purified phosphatase was subjected to cyanogen bromide cleavage to obtain internal polypeptide fragments for amino acid sequence analysis (Gross, 1967; Hirose, *et al.*, 1987). One mg of phosphatase, dissolved in 0.5 mL of 70% formic acid, was reacted at room temperature with a 500 molar excess of CNBr under nitrogen for 18 h. After addition of 9.5 mL of deionized water, the sample was lyophilized. The protein was redissolved in 1 mL of deionized water and stored in 250 μL aliquots. The CNBr fragments were analyzed on a 12% SDS polyacrylamide gel and stained with Coomassie Blue. The N-terminal sequences of the phosphatase and the CNBr fragments were determined by the Tripartite Microanalytical Centre at the University of Victoria, Victoria, British Columbia.

2.3.2 Determination of the carboxy-terminal amino acid sequence

The phosphatase was digested with carboxypeptidase P (Ambler, 1967, Yokoyama, *et al.*, 1975) in an attempt to identify amino acids at the carboxy-terminus of the enzyme. Approximately 90 nmol of the phosphatase was dissolved in 200 μL of 0.2 M pyridine/formate buffer, pH 4.2 and digested at room temperature with 90 μL (0.58 units) of carboxypeptidase P. Aliquots of 39 μL were removed after 0, 5 and 15 min, and after 1, 5 and 25 h. The reactions were stopped by the addition of 6 μL of 50% trifluoroacetic acid, frozen in a dry ice/ethanol bath and lyophilized. The samples were analyzed on a Beckman 6300 amino acid analyser (Beckman Instruments Inc., Palo Alto, CA) at the facility of the Medical Research Council Group in Protein Structure and Function, Department of Biochemistry, University of Alberta.

2.4 DNA Manipulations and Recombinant Methods

2.4.1 DNA isolation

Genomic DNA was prepared by suspending 1 g of wet cells in 10 mL of 25% sucrose, 50 mM EDTA, 0.5 mg/mL of lysozyme. After 10 min at room temperature, SDS was added to a concentration of 2% and the mixture was warmed to 60°C. The cell lysate was digested with 1 mg/mL of Proteinase K at 37°C for 18 h. The aqueous phase was extracted three times with an equal volume of phenol. A small volume of chloroform was added prior to centrifugation to improve phase separation. One mL of 3 M sodium acetate, pH 5.0 was added and the DNA was precipitated with 10 mL of isopropyl alcohol. The DNA was transferred with sterile forceps to another container and recentrifuged to remove excess liquid. The DNA was redissolved in TE buffer.

For recombinant lambda phage isolation, *E. coli* LE392 cells were grown in LB broth supplemented with 10 mM $MgCl_2$ for large-scale production of λ-DASH and recombinant phage. Phage particles in the supernatant were precipitated, purified by cesium chloride gradient centrifugation and dialyzed (Sambrook, *et al.*, 1989). DNA was liberated from the phage by digestion with 50 μg/mL of Proteinase K for 1 h at 60°C. Contaminating protein was removed by phenol/chloroform extraction and the DNA was precipitated in ethanol.

Plasmid DNA was purified using the alkaline plasmid preparation method (Birnboim and Doly, 1979). For double-stranded plasmid sequencing, the plasmid preparation was precipitated in 13% PEG, 1 M NaCl for a minimum of 2 h at 0°C after treatment with RNase. Single-stranded DNA for dideoxy-sequencing was prepared according to the method of Vieira and Messing (1987) with minor modifications. A single colony of *E. coli* MV1193 containing the desired plasmid was grown in 4 mL of 2 x YT plus Amp overnight. Ten millilitres of medium was inoculated with 25 μL of the overnight culture and 100 µL of concentrated M13KO7 helper phage solution. After 75 min at 37°C, Kan was added to a final concentration of 70 µg/mL and incubation was continued overnight. Cells were removed by centrifugation and single-stranded phagemids were precipitated by the addition of 2.5 mL of 20% PEG, 2.5 M NaCl to the supernatant. The mixture was left at room temperature for 1 h and then centrifuged at 12 000 x g, 15 min, 0°C. The phage pellet was resuspended in TE buffer, phenol-extracted, precipitated in ethanol and dissolved in 50 µL of TE buffer. Usually, 2 µL of this solution was sufficient for one sequencing reaction.

M13KO7 helper phage concentrate was prepared by inoculating 10 mL of 2 x YT plus 70 µg/mL of Kan with a single plaque from an infected *E. coli* MV1193 plate. The culture was grown overnight at 37°C and used to inoculate 500 mL of fresh medium containing Kan and incubation was continued overnight. The supernatant containing the phage was kept and PEG was added to 4% and NaCl to 0.5 M. The solution was kept on ice overnight and centrifuged. The pellet was resuspended in 50 mL of SM buffer, incubated at 60°C for 30 min and stored at 4°C.

2.4.2 Preparation of probes

The oligonucleotide probes shown in Table 2-1 were used in DNA hybridization studies, DNA sequencing or primer extension. They were prepared by end-labelling with 10 µCi γ-^{32}P-ATP using T4 polynucleotide kinase (Southern, 1975; Maxam and Gilbert, 1980). Fragments from digested recombinant plasmids or PCR products were melted from 1% low melting point agarose, phenol extracted and labelled by the random primer method (Feinberg and Vogelstein, 1983, 1984). Approximately 20 µCi α-^{32}P-dATP was used per preparation. These probes were passed through a 5 mL Sephadex G-25 or G-50 column equilibrated with TE plus 0.1% SDS to remove any of the unincorporated radioactive nucleotides.

2.4.3 Gel electrophoresis, Southern transfer and DNA hybridization

Genomic or plasmid DNA fragments were separated by agarose or polyacrylamide gel electrophoresis in TEA buffer, stained with ethidium bromide and photographed under UV light. An aperture of f8 and a shutter speed of 1 sec or 90 sec were selected for Polaroid 667 or 665, respectively. The DNA was transferred to Hybond-N nylon membranes based on the method of Southern (1979) as modified for nylon membranes (Reed and Mann, 1985; Rigaud, *et al.*, 1987). Gels were treated with 0.25 M HCl for 7 min, followed by alkaline denaturation in 0.5 M NaOH, 1.5 M NaCl for 30 min and finally in 1 M NaOAc, 10 mM NaOH for 30 min. The DNA was transferred to the membrane in the same solution. The membranes were rinsed in 2 x SSPE, air dried and baked under vacuum for 2 h at 70°C.

Plaque lifts were performed by laying nitrocellulose or nylon onto cooled overnight plate cultures of λ-infected *E. coli* LE392 for 1 min. The phage DNA on the membrane was released by treatment with 0.15 M NaOH, 1.5 M NaCl, for 1 min and neutralized in 3 M NaOAc, pH 5.5 for 5 min. The membranes were rinsed in 2 x SSPE, dried and baked (Benton and Davis, 1977).

Colony lifts were prepared by growing plasmid containing *E. coli* MV1193 cells in duplicate on 2 x YT plates plus Amp, one of which had a Millipore HATF nitrocellulose filter on the agar surface. The cultures were incubated overnight at 37°C. The cells on the filter were lysed with 0.5 M NaOH for 7 min, neutralized with 1 M Tris/HCl, pH 7.5 twice for 5 min and finally in 0.5 M Tris/HCl, pH 7.5, 1.5 M NaCl. After baking, the filters were rehydrated in 2 x SSPE plus 0.1% SDS at 60°C and the cell debris was scraped off (Grunstein and Hogness, 1975).

All membranes were prehybridized at 60°C with hybridization solution [6 x SSPE, 5 x Denhardt's solution (Denhardt, 1966), 0.5% SDS] and 100 µg/mL of sonicated salmon testes DNA in a Seal-a-Meal™ bag for a minimum of 3 h on a rotisserie apparatus. The prehybridization solution was replaced with fresh hybridization solution containing the ^{32}P-labelled probe and up to 60% deionized formamide, depending on the desired stringency and the degeneracy of the probe. Incubation continued for a minimum of 12 h at 45°C. The filters were washed with 2 x SSPE plus 0.1% SDS at 60°C for 2 x 30 min, wrapped in plastic wrap and used to expose Kodak XAR5 or Fuji X-ray film with a Dupont Cronex Lightning Plus or a Quanta III intensifying screen at -70°C.

2.4.4 Polymerase chain reaction

Various primers were synthesized for the generation of modified DNA fragments by PCR. Table 2-3 lists primers with the various alterations made to *phoA* and Table 2-4 lists the PCR products generated for use in the preparation of recombinant vectors for expression experiments. Between 1-5 ng of single-stranded DNA of pSA3 was dissolved in PCR reaction buffer containing a final concentration of 50 mM Tris/HCl, pH 9.0, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 10% DMSO, 250 µM each dNTP and up to 0.25 µM of each primer. The PCR reactions were overlaid with mineral oil and incubated on a thermal cycler for 25 cycles of 30 sec at 94°C, 1 min at 60°C and 3 min at 72°C (Saiki, *et al.*, 1988). The mineral oil was removed from the reaction mixture by chloroform extraction or adsorption to Parafilm™ prior to precipitation in isopropyl alcohol and dissolution in TE buffer.

Table 2-3: Modifications made in PCR primers for the expression of the phosphatase gene in *E. coli*[a,b].

name of primer	sequence
	5' wild-type sequence
	267 311
	..CCACTCCCGCCGGAGACAGCCAGTGAACCTCTCGCCCTCGCGCAC...
SAU14	CAGTGAACCTCTCGCCCTCGCGCAC...
SAU19	CTCGAATTCAGGAGACAGCCATATGAACCTCTCG
SAU27	CAATGAATTCGTCGCCCTCGCGCAC...
	wild-type signal peptide and propeptide region
	368 410
	...CGCACGCCGCCCAGCGATCCTGCAGCTGTCGGAGGACACCAC...
SAU29	GCCGGAATTCCATCCTGCAGCTGTCGGAGGACACC
	mature protein region
	721 773
	...GCGCTGGACAAGGCCACGGTGGTCGTGGCCGGCGCCGGCGACA...
SAU24	CGTCGAATTCGGCCACGGTGGTCGTGGCCGGC
	3' wild-type sequence
	1977 1935
	...TCCACGCCGTCGCTGGAGGTCGAAGACAGCACCACGCCGTCGT...
SAU13	CCGTCGCTCGAGGTCGAAGACAGC
SAU30	CGGCGAATTCCGTCGCTCGAGGTCGAAGACAGC

*position numbers are indicated according to the sequence shown in Figure 3-2.

[a] incorporated restriction sites are underlined.

[b] Shine-Dalgarno sequences are dotted underlined.

Table 2-4: PCR products generated for cloning into expression vectors.

primers upstream/downstream	name	purpose/features
SAU14/SAU13	WT	-wild-type alkaline phosphatase gene
SAU19/SAU30	M3	-modified phosphatase gene
SAU24/SAU30	Mat	-no signal peptide or propeptide
SAU27/SAU30	-SD	-no Shine-Dalgarno sequence
SAU29/SAU30	PP	-no signal peptide

2.5 The Cloning of the Phosphatase Gene

2.5.1 Preparation of λ packaging extract

Lambda packaging extract was prepared by the method of Rosenberg (1987). Four flasks containing 115 mL of LBK medium were inoculated with an overnight culture of *E. coli* SMR10 grown at 34°C and incubated at the same temperature. When an OD_{550} of 0.80 was reached, the cultures were pooled into two flasks and incubated with vigorous aeration at 44°C for 15 min before the temperature was reduced to 37°C for 90 min. The cultures were chilled on ice, centrifuged and each pellet was suspended in 4.5 mL of TSP buffer, pooled and recentrifuged as above. The pellet was resuspended in 0.35 mL of TSP. Aliquots of 20 µL were removed and mixed with 5 µL of a solution containing 50% DMSO and 75 µM ATP and frozen uncapped in liquid nitrogen. When required, a tube of extract was thawed on ice after which an overnight ligation reaction containing substrate DNA was added and mixed. The reaction was allowed to proceed at room temperature for 2 h, after which 0.5 mL of phage dilution buffer and 25 µL of chloroform was added and mixed by vortexing. A portion of the packaged phage was serially diluted to 1×10^{-3}, 1×10^{-4} and 1×10^{-5}, and aliquotes of 100 µL were used to infect 100 µL of freshly grown *E. coli* LE392 indicator bacteria. After adsorption for 30 min at 37°C, 3 mL of soft LB agarose was added and the mixture was overlayed onto LB plates and incubated overnight at 37°C.

2.5.2 Production of a genomic libary in λ-DASH

A partial digest of *L. enzymogenes* genomic DNA was prepared using *Mbo*I in order to obtain 12-20 kb fragments which were fractionated by sucrose gradient centrifugation (Sambrook, *et al.*, 1989). λ-DASH DNA was digested with *Bam*HI and

*Xho*I and the small fragments were removed by polyethylene glycol precipitation (Lis, 1980). The phage arms and the partially digested genomic DNA were ligated in an approximately 3:1 ratio and packaged using λ packaging extract to produce a genomic library (Rosenberg, 1987). *E. coli* LE392 cells were infected with a sample of the phage library, plated onto 22 x 22 cm LB agar plates and incubated overnight at 37°C. The plaques were lifted and transferred onto nylon membranes and probed as described above. Plaques that hybridized to the probes were selected and the phage therein were used to grow 1 L of phage culture for a large-scale purification (Sambrook, *et al.*, 1989). Purified recombinant phage DNA was digested with various restriction enzymes and subcloned into pUC118 or pUC119 to obtain smaller cloned DNA fragments and for single-stranded DNA sequencing.

2.5.3 DNA sequencing

The dideoxy chain termination method was used to sequence the single-stranded phagemid DNA. Labelling of extension products was achieved by the incorporation of [α-^{32}P]-dATP (Sanger, *et al.*, 1977). The Klenow fragment of *E. coli* DNA polymerase was used in the initial sequencing experiments. Subsequently, Sequenase™ or T7 DNA polymerase was employed (Tabor and Richardson, 1987). In order to minimize compressions, 7-deaza-dGTP was substituted for dGTP in the Sequenase™ labelling and termination mixes (Mizusawa, *et al.*, 1986). DMSO was added to a final concentration of 10% to minimize premature stopping of chain elongation (Winship, 1989). Double-stranded sequencing was accomplished using Taq DNA polymerase (Promega Protocols and Applications Guide). Double-stranded DNA was denatured in 0.2 M NaOH, 0.2 mM EDTA for 7 min and precipitated with 3 M NaOAc, pH 5.0 and ethanol prior to the labelling reaction. The reactions were separated on 6% denaturing polyacrylamide gels using TEB buffer on a BRL model S1 sequencing apparatus (Smith and Calvo, 1980).

Labelled DNA bands were visualized by autofluorography. Sequencing data were interpreted independently by at least two people to minimize reading errors. Sequencing primers were synthesized as required to extend the sequence.

2.5.4 Isolation of total RNA and primer extension

L. enzymogenes total RNA was isolated from phosphatase-producing stationary phase cultures using the hot phenol method (Sambrook, *et al.*, 1989). A 1.5 mL volume of culture was centrifuged and the pellet was resuspended in 100 µL of lysis buffer. An equal volume of TE-equilibrated phenol was added, mixed vigorously by vortexing and incubated at 65°C for 10 min with intermittent mixing. An equal volume of DEPC-treated water and 50 µL of chloroform was added to improve phase separation. After centrifugation, the aqueous layer was transferred to a sterile 1.5 mL tube and precipitated in 10 µL of 3 M NaOAc, 250 µL of isopropyl alcohol and stored at -20°C until required.

Approximately 80 µg of RNA was dissolved in a total of 9.5 µL with 12.5 ng of ^{32}P end-labelled primer (5'-GTTCACTGGCTGTCTCC-3'), reverse transcriptase buffer, 0.5 mM of each dNTP and 0.2 mg/mL of actinomycin D in preparation for the primer extension reaction (Calzon, *et al.*, 1987). One half of a µL containing nine units of AMV reverse transcriptase was added and the mixture incubated for 1 h at 45°C. The reaction was stopped by placing the reaction at 90°C for 1 min and cooling on ice. Ten µg of RNase was added and incubation continued for 30 min at 37°C. After phenol/chloroform extraction, the primer extension products were precipitated with ethanol using 5 µg of tRNA as carrier. Products of the extension reaction were separated on a 6% denaturing polyacrylamide gel alongside a sequence ladder which utilized the identical primer.

2.6 Gene expression

2.6.1 Construction of recombinant expression plasmids

Primers flanking the phosphatase gene were synthesized such that the upstream primers contained a useful restriction site such as *Eco*RI, a consensus ribosome binding site and a GTG to ATG alteration of the initiator codon. The downstream primer was constructed with *Xho*I and *Eco*RI sites to facilitate cloning. The PCR products were prepared as described above and used to clone into the various expression vectors. pT7-3 was used as a positive control since it contains a β-lactamase gene that is under the control of the T7 promotor (Tabor and Richardson, 1985). The presence of a 30 kDa band in rifampicin-containing samples would demonstrate the induction of the T7 RNA polymerase and synthesis of the β-lactamase. The PCR product M3, containing the modified phosphatase gene, was cloned into the *Nde*I-*Sal*I sites of pT7-7 and the *Eco*RI-*Xho*I sites of pBluescriptIIKS$^+$ so that transcription would be controlled by a T7 promotor and the T7 RNA polymerase. These constructs were named pT77M3 and pKSM3, respectively. Translation was dependent on the *E. coli* ribosome binding site already existing in pT77 for pT77M3 or by the consensus ribosome binding site incorporated into M3 for pKSM3. M3 was also cloned into the *Eco*RI-*Sal*I sites of pUC118 and pKK223-3 which have IPTG inducible promotors and were called pES10 and pKKM3, respectively. These clones were sequenced to verify the identity of the vector and the insert prior to the expression experiments.

2.6.2 *In vivo*-labelling of expressed proteins

The procedure was based on the method developed by Tabor and Richardson (1985). A single colony of either *E. coli* K38/pGP1-2 grown at 30°C or *E. coli* JM109

(DE3) grown at 37°C containing the T7 promotor-controlled gene was grown overnight in modified LB broth with the appropriate antibiotic(s). The overnight culture was used to inoculate 10 mL of fresh medium which was allowed to grow to an OD_{600} of 0.5. Three 1 mL samples of culture were pelleted and washed twice by resuspension with 5 mL of minimal medium and resuspended in 5 mL of minimal medium supplemented with 0.02% of 18 amino acids, not including cysteine or methionine. Incubation was continued at the appropriate temperature for 1 h. Cells were induced by shaking at 42°C (K38/pGP1-2) or by the addition of IPTG to 0.5 mM (DE3) for 20 min. Rifampicin, an inhibitor of host RNA polymerase, was added to 200 µg/mL and incubation was continued for an additional 30 min. For K38/pGP1-2, the incubation temperature was reduced to 30°C 10 min after the addition of rifampicin and incubation was continued for 20 min. The cells were pulse-labeled with 10 µCi ^{35}S-methionine for 5 min, centrifuged for 30 sec and the pellet was resuspended in 150 µL of 10 mM Tris/HCl, pH 8.0, 0.1% β-mercaptoethanol, 10% glycerol, 0.01% Bromphenol Blue and heated to 90°C for 10 min. Samples were separated by SDS-PAGE and stained with Coomassie Blue. After photography, the gel was dried onto Whatmann 3MM paper and used to expose X-ray film. Plasmids from the overnight inoculum were isolated and their sizes were reconfirmed by agarose gel electrophoresis.

2.6.3 Expression and partial purification of the secreted phosphatase from *E. coli*

Cells containing the appropriate plasmid were grown overnight in modified LB broth plus 150 µg/mL Amp. The overnight cultures were used to inoculate 200 mL of medium and incubated until an OD_{600} of about 1.0 was reached. The cultures were induced with IPTG at a final concentration of 0.5 mM and incubation was continued for 2.5 h. The shock fluid was obtained using a method based on that of Neu and Heppel (1965). The cultures were centrifuged at 4 000 x g, 22°C for 10 min. The cells were washed twice in 200 mL of 30 mM Tris/HCl, pH 8.0 and resuspended in 0.5 mL of 30 mM Tris/HCl, pH 8.0, 2 mM EDTA and 25% sucrose and incubated at room temperature for 10 min. The cell mixture was forcefully pipetted into 200 mL of deionized water at 0°C and mixed on a magnetic stirrer for 15 min. The shocked cells were centrifuged at 20 000 x g for 15 min at 0°C and the supernatant was frozen in dry ice/ethanol and lyophilized. The residue was redissolved in 3 mL of cold deionized water. A 2.5 mL sample was applied to a Sephadex G-75 Superfine column and eluted as previously described. Protein concentrations were measured by A_{280}. Fractions were pooled as described in Table 2-5, dialyzed against 10 mM Tris/HCl, pH 7.5, lyophilized and redissolved to 1 mL with deionized water. Samples of whole cells, shock fluid, supernatant and pooled Sephadex fractions were analyzed by SDS-PAGE and stained with Coomassie Blue and/or transferred to nylon membranes for immunological detection.

2.6.4 Western transfer and immunoblotting

Proteins on SDS-polyacrylamide gels were transferred to nylon membranes based on previously developed procedures (Towbin, *et al.*, 1979; Burnette, 1981). The gel was soaked in tank buffer (Table 2-2) for 30 min following electrophoresis. The gel was then layered between one sheet of nylon membrane (pre-wetted) and two sheets of Whatman 3MM paper cut to size and mounted onto the transfer apparatus. The proteins were transferred in a chamber containing tank buffer with the positive electrode on the same side as the membrane. Electrotransfer was performed at 0°C using 0.2 mA for 2 h. The membrane was air-dried after rinsing in distilled water for 10 min.

The membrane was pre-incubated at 37°C with 20 mL of Solution I (Table 2-2) for 2 h in a Seal-a-Meal™ bag. Five mL of solution I plus 0.5 mL of undiluted antiserum was exchanged for the previous solution and incubation continued overnight. The membrane was rinsed with 20 mL of fresh solution I for 1 h and then exchanged for 5 mL of solution I plus a small volume containing 0.5 μCi ^{125}I-protein A. Incubation was continued for a

Table 2-5: Preparation of pooled Sephadex fractions.

pool	contents/description
Seph. I	fractions 15-25, void volume/negative control
Seph. II	fractions 36-42, elution region of blue dextran (V_0)
Seph. III	fractions 43-51, elution region of OVA
Seph. IV	fractions 52-58
Seph. V	fractions 59-65
Seph. VI	fractions 66-72, elution region of the secreted phosphatase
Seph. VII	fractions 73-79, part of elution region of cytochrome C
Seph. VIII	fractions 80-86, part of elution region of cytochrome C
Seph. IX	fractions 87-98
Seph. X	fractions 99-110, elution region of NH_4^+ (V_t)

minimum of 4 h. The membrane was rinsed in 20 mL of solution II (Table 2-2) for a minimum of 1 h at 37°C, wrapped in plastic wrap and used to expose X-ray film at -70°C.

2.7 Analytical Methods

2.7.1 Quantification of proteins and nucleic acids

Protein concentrations were determined by two spectrophotometric and one colorimetric methods. The A_{280}, A_{260} and A_{230} of a suitably diluted protein sample were measured and the concentrations were calculated by using the appropriate conversions developed by Warburg and Christian (1941) or Kalb and Bernlohr (1977). The Bradford assay was performed for a comparative measurement. Up to 20 µg of protein was dissolved in 0.8 mL of water after which 0.2 mL of Bio-Rad solution concentrate was added (Bio-Rad Laboratories, Richmond, CA). After incubation at room temperature for 10 min, the absorbance was read at 595 nm. Bovine gamma globulin was used as a protein standard.

The concentration of nucleic acids was determined by measuring the absorbance of a suitably diluted sample at 260 nm where 1.0 A_{260} is approximately 35 µg/mL for oligodeoxyribonucleotides, 50 µg/mL for double-stranded DNA and 40 µg/mL for single-stranded DNA and RNA (New England Biolabs Catalog, 1992).

2.7.2 Detection of iron by atomic absorption

Atomic absorption of iron was analyzed in Dr. Dudas' laboratory, Department of Soil Science, Univerisity of Alberta, using an Instrumentation Laboratory aa/ae spectrophotometer 751 with an iron lamp. Absorbance was measured at 248.3 nm using a lean-blue air-acetylene flame with 4 sec integration. A standard solution of 0.989 mg Fe/mL was prepared by dissolving 0.50 g of 99.9% iron wire in 25 mL of HNO_3 and 15 mL of HCl and diluting to 500 mL with deionized water. Standards of 0.0, 0.5, 1.0, 3.0 and 5.0 μg of Fe were used to calibrate the spectrophotometer. Purified phosphatase was dissolved to 2 mg/mL and 1 mg/mL as measured by A_{280}/A_{260}.

2.7.3 β-lactamase assay

β-lactamase activity was determined by pipetting 50 μL of enzyme sample into 850 μL of 0.1 M Tris/HCl, pH 7.5 and 100 μL of 0.1 mM PADAC at 22°C. The decrease of the A_{570} was measured and traced on a recorder for 2 min. When required, enzyme samples were diluted appropriately with 0.1 M Tris/HCl, pH 7.5, 1 mg/mL of BSA. One unit of β-lactamase activity is the amount of enzyme which hydrolyzes 1 μmol of PADAC per min at 22°C. An extinction coefficient of 52 700 for PADAC was used to calculate enzyme activity.

2.7.4 Computer software and sequence analysis

The FRAME program for %G+C analysis (Bibb, *et al.*, 1984) adapted for the Macintosh was provided by Dr. S.E. Jensen. The %G+C content for each codon position was calculated using a window of 120 nucleotides. DNA sequence analysis was performed using DNA Strider 1.0 (Marck, 1988). PC-GENE was used to determine possible signal sequence cleavage sites. The Mail-Fasta [EMBL File server] (Pearson and Lipman, 1988), was used to search the current Swiss-Prot and PIR databases for amino acid sequence similarities between the phosphatase and other proteins.

CHAPTER 3

Results

3.1 Characterization of the phosphatase

3.1.1 Purification and CNBr digestion of the phosphatase

The phosphatase was isolated as described in Materials and Methods, Section 2.2.2. and its purity was analyzed by SDS-PAGE (not shown). Many properties of the enzyme have been reported earlier (von Tigerstrom, 1984). In this study, it was found that the protein band corresponding to the phosphatase migrated slightly behind the carbonic anhydrase marker (M_r 28 980). Therefore it was estimated to have a molecular weight of about 30 kDa. Three methods were used to obtain an estimate of protein concentrations of the purified enzyme preparation. It was important to have an accurate determination of the amount of protein in the purified preparation for the iron determination. Quantification of protein using A_{280}/A_{260} measurements (Warburg and Christian, 1941) and A_{260}/A_{230} measurements indicated concentrations of 1.85 mg/mL and 1.40 mg/mL, respectively. The deduced amino acid sequence of the phosphatase revealed that the protein contained a disproportionately high amount of aromatic amino acids, such as tyrosine or tryptophan, which probably resulted in an overestimation of the protein concentration. Therefore, subsequent estimates were divided by two to account for this. The Bio-Rad assay using bovine gamma globulin as a standard protein gave a value of 1.15 mg/mL, about 60% of the A_{280}/A_{260} measurement. The specific activity of the purified enzyme was found to be approximately 175 units/mg of protein, using the Bio-Rad determinations.

The CNBr degradation of the purified enzyme produced one large fragment and two smaller fragments which have an apparent molecular weight of approximately 9 kDa each. On an SDS-polyacrylamide gel, the smaller CNBr fragments were very faint and difficult to resolve. Samples of the purified phosphatase and the CNBr fragments were lyophilized for N-terminal amino acid sequence analysis. The amino acid sequences of the N-termini of the mature enzyme and the two small CNBr fragments are listed in Table 3-1. The large CNBr fragment (not shown) was found to have an amino acid sequence identical to the mature N-terminus. As indicated below, the smaller fragments were found to be located within the sequence of the mature phosphatase. This information was used to construct oligonucleotide probes for screening recombinant phage and plasmid isolates for the phosphatase gene.

3.1.2 Production of anti-alkaline phosphatase antibody

Anti-AP was produced in rabbits challenged with the secreted phosphatase over a period of about 7 months. The extended period of time was required because permission to use the Freund's adjuvant system was given reluctantly by the director of the Biosciences Animal Service. The titres of antiserum from blood samples during the course of the immunization were poor and the maximum dilution that permitted observation of significant enzyme inhibition never reached more than 1:1000. However, inhibition was specific enough to differentiate between *L. enzymogenes* cell-associated phosphatase, *E. coli* phosphatase and the secreted phosphatase from *L. enzymogenes* B-13 (data not shown). This preparation of antiserum was subsequently used to detect recombinant phosphatase activity and for immunoblot experiments using of samples from gene expression experiments.

Table 3-1: N-terminal amino acid sequences of the *L. enzymogenes* mature secreted phosphatase and CNBr fragments[a].

amino terminus		**CNBr fragment #1**	**CNBr fragment#2**
amino acid sequence	amino acid sequence (cont)	amino acid sequence	amino acid sequence
Ala (GCG/C)[c]	Thr	Met[d]	Met[d]
Thr (ACC)	Ser	Asn	Ser
Val (GTG/C)	Asp	Pro	Gly
Val (GTG/C)	Leu	Asp	Gly
Val (GTG/C)	Ile	Thr[b]	Lys[b]
Ala (GCG/C)	Val	Ala	Val
Gly (GGC)	Thr?	Ala	Ala
Ala (GCG/C)	Ile	Ala	Gln
Gly (GGC)	Asn	Ser	Ala
Asp (GAC)	Pro	Asp	Gln
Ile (ATC)		Gly	Ile
?		Cys	
Asp		Gln	
Thr		Val	
Ser		Ser	
Gly		Val	
Asn		Gly	
Ala		Thr	
?		Gly	
Gln		Gly	
Gly			

[a] The amino acid residues are those obtained from the amino acid sequencing analysis.

[b] These two residues were reversed in the amino acid sequence analysis. This error may have occured since the sequence for fragment #2 was a mixture and was deduced by subtraction of residues from fragment #1.

[c] The codons used for the synthesis of the oligonucleotide probe are shown in parentheses.

[d] The methionine residues for the CNBr fragments are inferred.

3.2 Isolation of the Phosphatase Gene

3.2.1 Cloning and sequencing of *phoA*

L. enzymogenes DNA was digested with a variety of restriction endonucleases including *Bam*HI, *Cla*I, *Eco*RI, *Hind*III, *Sal*I and combinations of these enzymes. The products were fractionated by agarose gel electrophoresis, transferred to nylon membranes and hybridized to the N-terminal probe of the purified phosphatase. The information obtained was used to identify landmarks in order to facilitate the identification of recombinant clones during phage or plasmid isolation and DNA sequencing.

Since *Lysobacter* was reported to have a high G+C content of approximately 68-70% (Christensen and Cook, 1978), Gs and Cs were used in the 'wobble' position of degenerate codons for the synthesis of the first oligonucleotide phosphatase gene probe as indicated in Table 3-1.

Fragments of an *Mbo*I partial digest of *L. enzymogenes* genomic DNA were cloned into λ-DASH and packaged to produce an *L. enzymogenes* genomic DNA library. Plaques were obtained as described in Materials and Methods, Section 2.4.3. One recombinant clone hybridized to the N-terminal probe and was amplified to obtain enough DNA for restriction analysis and subcloning experiments. The recombinant phage was called λLEM1 (***L. enzymogenes*, *Mbo*I**) and it contained an insert of approximately 12 kb. λLEM1 was cut with *Cla*I and yielded two large products of greater than 12 kb and a smaller fragment of about 1 kb. Two fragments, including the 1 kb fragment, hybridized strongly to the probe. An earlier restriction endonuclease analysis of *L. enzymogenes* genomic DNA also identified a 1 kb *Cla*I fragment which hybridized to the same probe. This observation was eventually explained by the existence of a G^{m}ATC methylation site overlapping the *Cla*I resistriction site (ie.GATCGAT) which prevented cutting of DNA isolated from *E. coli* by *Cla*I. During the large-scale preparation of the DNA, apparently not all of the phage DNA molecules were modified by a methylase equally. Therefore, some of the recombinant λLEM DNA were only cut once by *Cla*I.

The λ-LEM DNA was digested with both *Eco*RI and *Bam*HI and the fragments obtained were cloned into pUC119 treated with the same enzymes. White colonies, which were β-galactosidase negative and therefore contained a DNA insert, were selected and the plasmids were screened by Southern hybridization using oligonucleotide probes. One clone, called pSA3, possessed an approximately 4.4 kb fragment from *L. enzymogenes* genomic DNA, which could be released by cutting with *Bam*HI and *Eco*RI. Due to the size of the fragment, it was probable that the sequence of interest was a significant distance away from the binding sites of the universal and reverse sequencing primers. Therefore, pSA3 was digested to completion with either *Alu*I or *Sau*3AI and the fragments were analyzed by polyacrylamide gel electrophoresis, Southern transfer and hybridization. The DNA fragments containing phosphatase sequences were about 350 bases in length. They were eluted from the gel (Maxam and Gilbert, 1980) and cloned in both directions into the *Hinc*II and *Bam*HI sites of pUC119, respectively, to obtain pSAA1, pSAA4, pSAS4 and pSAS44. Subsequently, a *Sal*I digest of pSA3 was cloned into pUC119 in both directions to produce pSAL4 and pSAL44. *Sal*I and*Cla*I digests of λLEM were also cloned in both directions into the *Sal*I and *Acc*I sites of pUC119, respectively to obtain pSSA, pCSA and pCSAi, an inverted pCSA fragment in pUC118. Table 3-2 lists the plasmids used for DNA sequencing and Figure 3-1 shows the sequencing stategy and a partial restriction map of the secreted phosphatase gene. Sequencing of these clones identified the nucleic acid sequence corresponding to the N-terminus of the mature phosphatase, the N-terminus of the precursor protein, approximately 40% of the mature amino acid sequence and the 5′ flanking region. As nucleotide sequence data accumulated, unique primers (Table 2-1) were synthesized to extend the sequence by primer walking in both directions. The nucleotide sequence for the secreted alkaline phosphatase gene, *phoA*, is shown in Figure 3-2 and has been assigned the EMBL accession number X56656.

Table 3-2 Characteristics of plasmids used for DNA sequencing of *phoA*.

Name of plasmid	enzyme(s) used for cloning	size	direction of sequence relative to gene
pSA3	*Eco*RI-*Bam*HI	≈4.4 kb	5'-3' @ about position -250[a]
pSAA1	*Alu*I	353 bp	3'-5' @ position 1046[b]
pSAA4	*Alu*I	353 bp	5'-3' @ position 693
pSAS4	*Sau*3A1	329 bp	3'-5' @ position 808
pSAS44	*Sau*3A1	329 bp	5'-3' @ position 479
pSAL4	*Sal*I	>2 kb	3'-5'
pSAL44	*Sal*I	>2 kb	5'-3' @ position 651
pSSA4	*Sal*I	>1 kb	3'-5' @ position 651
pCSA6	*Cla*I	1 112 bp	3'-5' @ position 1119
pCSAi	*Cla*I	1 112 bp	5'-3' @ position 7

[a] sequence data 5' to the *Eco*RV site are not reported

[b] positions are numbered as in Figure 3-2.

Figure 3-1: Sequencing strategy and map of the secreted phosphatase gene. The arrows below the diagram indicate the direction and extent of sequence obtained using subclones of pSA3. The arrows above indicate the direction and extent of sequence obtained using unique 17-base oligonucleotide primers. Some restriction sites and key features of the phosphatase are highlighted. The shaded region is drawn to scale.

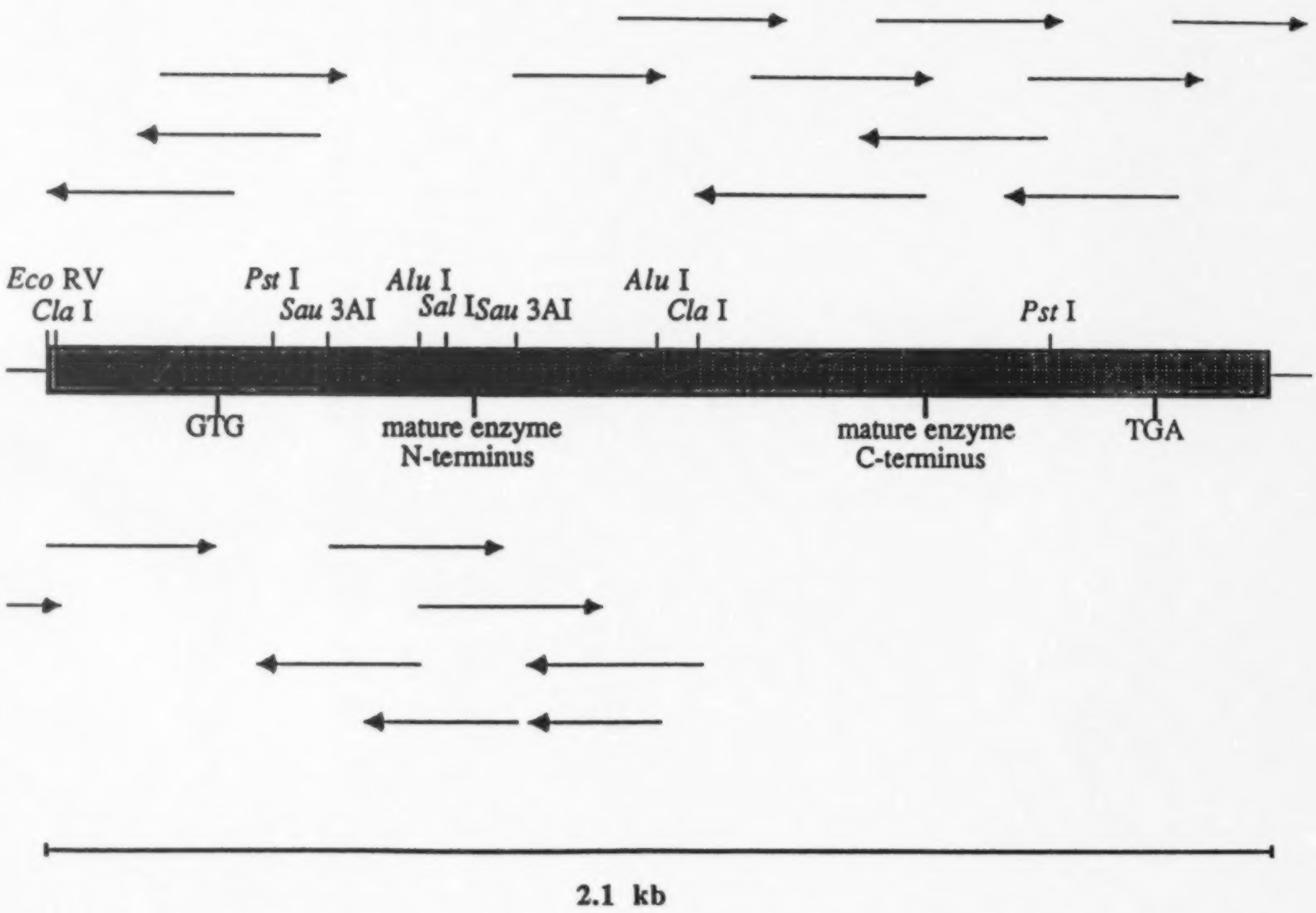

Eco RV
Cla I
Pst I
Sau 3AI
Alu I
Sal I
Sau 3AI
Alu I
Cla I
Pst I
GTG
mature enzyme N-terminus
mature enzyme C-terminus
TGA
2.1 kb

Figure. 3-2: Nucleotide sequence of *phoA* and the deduced amino acid sequence of the secreted phosphatase. The nucleotide sequence of a 2292 bp region which contains the phosphatase gene is shown beginning from an *Eco* RV site. The 1620 bp open reading frame and the corresponding 539 amino acid sequence, numbered -148 to +391, is indicated. The first amino acid of the mature enzyme N-terminus is designated (+1) and the N-terminus of the prepro-peptide is assigned (-148). The first digit is aligned beneath the corresponding residue. The putative Shine-Dalgarno box is indicated by dotted underline and the N-terminal signal sequence is enclosed in square brackets. Amino acid sequences obtained from the two cyanogen bromide fragments are double-underlined. Two possible transcriptional terminators are underlined. The asterisk denotes the transcription start site as determined by primer extension. Restriction sites used for cloning are listed in bold-type.

```
EcoR V/Cla I Sph I
TGATATCGATAAGCATGCGTTCTTTACACACGAAACGTGAGGCCGCAGGCCGCGCCCGCT      60
TGTGCGCCTCGCGCGTGGGCGCGCCCGTCTGCGGCGGTGATGCGAGCGCTGCGGCGAAGA     120
CAAGTCGGAACTTGTGACGGACGCCGCGCTGACGGCGCAACAGCCGCGCATTGCGTCGCA     180
ATCGTGTAGCAGTTTTCCGGCCAAACTCCGCAGCCATCGCGGCCTTGCCCGGCCGCGATG     240
CCCGCCTTCATCCGCCCCGCTACCAGCCACTCCCGCCGGAGACAGCCAGTGAACCTCTCG     300
                      *                        [fM N  L  S
                                               (-148)
             Sau3A I
CCCTCGCGCACGCCGATCTGCGCCGCCCTCGCCGCCGCCCTGCTCGGCGCCGCCGCGCTC     360
P  S  R  T  P  I  C  A  A  L  A  A  A  L  L  G  A  A  A  L
          (-140)
                           Pst I
GCGCCCGCGCACGCCGCCCAGCGCATCCTGCAGCTGTCGGAGGACACCACCCACAGCAAG     420
A  P  A  H  A] A  Q  R  I  L  Q  L  S  E  D  T  T  H  S  K
          (-120)
                                       Bal I             Sau3A I
CCGGTCAGCGCCGCCTCCGCGTTGCGCGGCACGCCGCTGGCCAAGGCCGGCGCCGCGGAT     480
P  V  S  A  A  S  A  L  R  G  T  P  L  A  K  A  G  A  A  D
          (-100)
                              Mlu I
CGCGTGTGCGAAGCCGGGGCCAAGTGGCTACGCGTGGGCTTCAAGCAGCTCAAGCTGGCC     540
R  V  C  E  A  G  A  K  W  L  R  V  G  F  K  Q  L  K  L  A
          (-80)
                                                 BstB I
GGCTACGACTCGCTGGTGCTGACCAGCAGCGGCGGCGACAAGCTCGTGTTCGAAGGCCAG     600
G  Y  D  S  L  V  L  T  S  S  G  G  D  K  L  V  F  E  G  Q
          (-60)
                                                  Sal I
CACTGGAACCAGCGCAGCTTCACCACCCGCCCGCTGCGCGGCGAGTGCGTCGACATCCAG     660
H  W  N  Q  R  S  F  T  T  R  P  L  R  G  E  C  V  D  I  Q
          (-40)
                                Alu I
CCGTATTTCAGCCAGCCCGACAGCGCCTTCCAGCTCGACCGCTACGACTACAGCACGGTC     720
P  Y  F  S  Q  P  D  S  A  F  Q  L  D  R  Y  D  Y  S  T  V
          (-20)
GCGCTGGACAAGGCCACGGTGGTCGTGGCCGGCGCCGGCGACATCTGCGACACCAGCGGC     780
A  L  D  K  A  T  V  V  V  A  G  A  G  D  I  C  D  T  S  G
          (+1)
                           Sau3A I Sau3A I
AACGCCTGCCAGGGCACCTCGGACCTGATCGTCTCGATCAACCCTACCGCGGTGTTCACC     840
N  A  C  Q  G  T  S  D  L  I  V  S  I  N  P  T  A  V  F  T
        (+20)
GCCGGCGACAACGCCTACAACAGCGGCACGCTGAGCGAATACAACAGCCGCTACGCGCCG     900
A  G  D  N  A  Y  N  S  G  T  L  S  E  Y  N  S  R  Y  A  P
        (+40)
ACCTGGGGCCGGTTCAAGGCGCTGACCAGCCCGTCGCCGGGCAACCACGACTACAGCACC     960
T  W  G  R  F  K  A  L  T  S  P  S  P  G  N  H  D  Y  S  T
        (+60)
ACCGGCGCCAAGGGCTACTTCGACTACTTCAACGGCAGCGGCAACCAGACCGGCCCGGCC    1020
T  G  A  K  G  Y  F  D  Y  F  N  G  S  G  N  Q  T  G  P  A
        (+80)
                         Alu I (PvuII)
GGCGACCGCAGCAAGGGCTACTACAGCTGGGACGTCGGCGACTGGCACTTCGTCTCCCTC    1080
G  D  R  S  K  G  Y  Y  S  W  D  V  G  D  W  H  F  V  S  L
        (+100)
```

```
                                    Cla I
AACACCATGAGCGGCGGCACCGTTGCGCAAGCCCAGATCGATTGGCTCAAGGCCGACCTC    1140
N  T  M  S  G  G  T  V  A  Q  A  Q  I  D  W  L  K  A  D  L
       (+120)
GCCGCCAACACCAAGCCCTGCACCGCGGCCTATTTCCATCATCCGCTGCTCAGCCGCGGC    1200
A  A  N  T  K  P  C  T  A  A  Y  F  H  H  P  L  L  S  R  G
       (+140)
AGCTACTCGGGCTACAGCCAGGTCAAGCCGTTCTGGGACGCGCTTTACGCGGCCAAGGCC    1260
S  Y  S  G  Y  S  Q  V  K  P  F  W  D  A  L  Y  A  A  K  A
       (+160)
GACCTGGTGCTGGTCGGCCACGACCACAACTACCAGCGCTACGGCAAGATGAATCCCGAC    1320
D  L  V  L  V  G  H  D  H  N  Y  Q  R  Y  G  K  M  N  P  D
       (+180)
                                               Not I
AAGGCCGCGGCCAGCGACGGCATCCGCCAGGTGTTGGTCGGCACCGGCGGCCGCGCCTTC    1380
K  A  A  A  S  D  G  I  R  Q  V  L  V  G  T  G  G  R  A  F
       (+200)
TACGGCATCAGCGGCAGCCACGCGCTGCTGGAAGCCAGCAACGACAGCACCTTCGGCGTG    1440
Y  G  I  S  G  S  H  A  L  L  E  A  S  N  D  S  T  F  G  V
       (+220)
CTCAAGCTGACCTTGAGCGCGACCGGCTACACCGGCGACTTCGTGCCGCGCGCCGGCAGC    1500
L  K  L  T  L  S  A  T  G  Y  T  G  D  F  V  P  R  A  G  S
       (+240)
AGCTACACCGACCATTTCACCGGCACCTGCAACAAGGGCAGCGGCAACCCGCCGACCCAG    1560
S  Y  T  D  H  F  T  G  T  C  N  K  G  S  G  N  P  P  T  Q
       (+260)
ACGCTGACGCTCAACAGCGTGCGCGATGTGACGGTGAAGTCCGGCGGCAGCCGCGACAAC    1620
T  L  T  L  N  S  V  R  D  V  T  V  K  S  G  G  S  R  D  N
          (+281)
GGCGCCACGCTCTACGCCGACGGCAGCGACGGCGGCCAGGTGTTGCGCGGCCTGATGGCG    1680
G  A  T  L  Y  A  D  G  S  D  G  G  Q  V  L  R  G  L  M  A
       (+300)
                                                   Pst I
TGGAATGTGTCGTCCGCCGCGGGCAAGACCCTCACCGGCGCGCAGGTCAAGCTGCAGGTC    1740
W  N  V  S  S  A  A  G  K  T  L  T  G  A  Q  V  K  L  Q  V
       (+320)
AGCGACCGCTCCACCGGCACCTACGACCTGTACCGCGCCGGCGCTGCCTGGACCGAGGCC    1800
S  D  R  S  T  G  T  Y  D  L  Y  R  A  G  A  A  W  T  E  A
       (+340)
AACGCGAGCTACAGCGGCGTGAGCCTGGGCTCCAAGATCGGCTCGGTCGTGCCCAGCGCC    1860
N  A  S  Y  S  G  V  S  L  G  S  K  I  G  S  V  V  P  S  A
       (+360)
ACCGGTGCGCAATCCATCGCGCTCAATGCCGCGGGCTTCAGCTGGTGAAGGACTGGGCGT    1920
T  G  A  Q  S  I  A  L  N  A  A  G  F  S  W  -
       (+380)                          (+391)
CGGCGCGGTCGCCAACGACGGCGTGGTGCTGTCTTCGACCTCCAGCGACGGCGTGGATTG    1980
GTCCTCGCGCGAAGGGCCCAATCGCCGGCAGGCTGATCCTGACCTACACGCCCTGATTCG    2040
GCGTTTGATTCGGCGGCTTGAGCTTGTGCGCCGAAGACAAACGAAAACGCCCGCGCAATG    2100
CCGGGCGTTTTCGTTGCCGCGGACGCGCGGTCAGGCCATGGCTTGCAAGGCCGGGCGCGC    2160
GGCGTGGGTGCCCGCGCCGGCGCCGATCATCAGCTTGACCGATTCCTTGATGCGCTCGGC    2220
                           Pvu I
GCCGCCGAGCTTGACCGCGGCGATCAGCGATCGCCATGGTCTTGCTTCGTCGGCTGCTGG    2280
CGTCCCGAGATG                                                    2292
```

3.2.2 Carboxy-terminal sequencing of the phosphatase

It became evident during DNA sequencing that the gene was much larger than previously estimated, due to the presence of an N- and a C-terminal extension. Attempts were made to determine the amino acid sequence of the C-terminus of the purified phosphatase so that a peptide cleavage point and a more accurate molecular weight could be determined. Purified enzyme was digested with carboxypeptidase P and aliquots were removed and the reactions terminated at various times to obtain samples with progressively shorter phosphatase molecules and liberated amino acids. The results from the amino acid analyzer were difficult to interpret (Table 3-3). Histidine was a possible candidate for the C-terminal residue, since it appeared to be released in the highest quantity after 25 h. The nearest histidine residue occurs at position 261. Subsequent residues could not be aligned with the known amino acid sequence near His-261 residue to maintain a molecular weight close to 30 kDa. Unhydrolyzed protein is expected to have a retention time similar to that of histidine and could prove to be the major constituent of that peak. Assuming that the histidine peak was an artifact, it was still difficult to align the remaining residues at or near Asn-281. There was no discernable sequential release of amino acids throughout the time of digestion. Smaller amino acid peaks corresponding to alanine, leucine, arginine, serine, valine, aspartate, threonine, glycine, tyrosine, lysine, glutamate and phenylalanine are some of the residues that could exist at and around the C-terminus. Less stable residues such as cysteine and tryptophan were not present and were probably destroyed at the low pH of the reaction. Clearly, the results were inconclusive. The analysis was repeated several times without obtaining more useful data and they were not pursued any further.

Table 3-3: C-terminal amino acid analysis of the secreted phosphatase*.

residue	5 min	15 min	1 h	5 h	25 h
asp	0.58	0.87	1.35	2.42	4.24
thr	1.22	1.74	2.31	3.07	4.58
ser	2.11	2.99	3.52	4.89	6.16
glu	0.08	1.11	1.24	1.35	2.23
gly	0.59	1.06	1.49	2.55	4.74
ala	2.24	3.23	4.30	5.69	7.90
val	1.46	2.46	3.39	4.55	5.60
met	0.17	0.66	0.62	0.74	1.11
ile	0.98	1.22	1.30	1.75	2.20
leu	3.54	4.14	5.05	5.96	7.55
tyr	1.45	1.40	2.08	2.64	3.23
phe	1.03	1.55	1.65	2.03	2.57
his	0.29	0.61	0.65	1.55	10.5
lys	2.22	2.79	3.14	3.57	4.23
arg	2.64	3.22	3.61	4.34	8.13

* amount of amino acid released (nmol) after digestion for the specified time.

3.2.3 Characterization of phosphatase precursor

The structural gene contains an open reading frame encoding a 539-residue protein. Primer extension analysis indicated that the transcription start site begins at nucleotide position 263 (Figure 3-3). Six base pairs upstream from the GTG initiation codon is a near-consensus GGAGA ribosome binding site (Shine and Dalgarno, 1975). Two inverted repeats are seen on the 3' side of the TGA stop codon. A small 6 bp inverted repeat with a 15 bp loop and a 13 bp inverted repeat with a 7 bp loop are located about 70 and 170 nucleotides from the termination codon, respectively. The latter is likely the transcriptional terminator.

A typical signal sequence is seen at amino acid position -148 through position -120. It contains one arginine followed by a hydrophobic core consisting mostly of alanines and leucine residues. The structure of the signal sequence was analyzed by PCGENE. An examination of the putative signal sequence determined a score of 13.1 (von Heijne, 1986), which indicates that there is a high probability for the existence of a signal sequence. Furthermore, this region conforms to the (-3,-1) rule of von Heijne (1983) that predicts the probable location of the peptidase cleavage site occurs between the two alanine residues at positions -120 and -119. The signal sequences of three *L. enzymogenes* enzymes are compared in Table 3-4. They all contain arginine residues in their charged regions. The β-lactamase and the phosphatase contain predominantly alanine, leucine and glycine residues in the hydrophobic cores. Following the signal peptide of the phosphatase, there is a 119-residue N-terminal extension preceding the beginning of the mature enzyme.

The region numbered +1 to approximately +281 corresponds to the mature enzyme. The N-terminal sequences of the two CNBr fragments have been located within the gene, extending from position +120 to position +129 and from position +194 to position +213, respectively. Asn-281 is tentatively designated the carboxy-terminal residue of the mature enzyme based solely on molecular weight information, since the C-terminal sequencing

Figure 3-3: Determination of the transcription start site by primer extension. *L. enzymogenes* total RNA was isolated from phosphatase-producing cultures. The complementary strand was synthesized by AMV reverse transcriptase and separated on a 6% denaturing polyacrylamide gel (right-outermost lane) alongside a sequence ladder which utilized the identical primer.

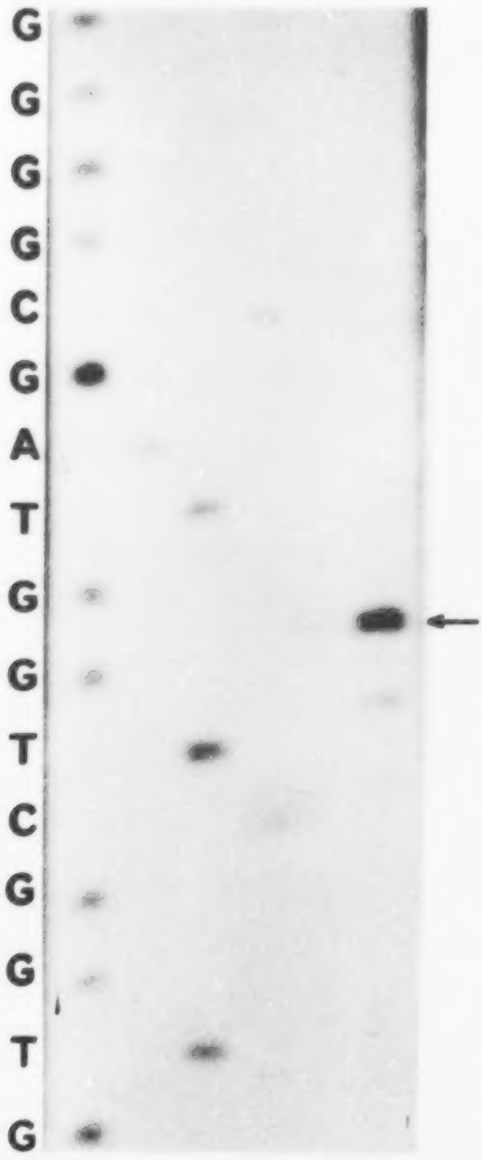

G
G
G
G
C
G
A
T
G
G
T
C
G
G
T
G

Table 3-4: Comparison of the signal sequences from the α-lytic protease, β-lactamase and the secreted phosphatase from *L. enzymogenes*[a,b,c].

enzyme	N-terminal amino acid sequence
α-lytic protease[d]	MYVSNHRSRRVARVSVSCLVAALA | ACPAALRWRP . . .
β-lactamase[e]	MQRRAFLQCTGSLLLAGGAVASFGAAA | LSPKPAA . . .
phosphatase[f]	MNLSPSRTPICAALAAALLGAAALAPAHA | AQRIL . . .

[a] charged residues are underlined.

[b] the hydrophobic core is double-underlined.

[c] '|' marks the peptidase cleavage site.

[d] Epstein and Wensink, 1987.

[e] Boras, *et al.*, 1993.

[f] this study.

experiments were inconclusive. The actual C-terminal amino acid may be up to 10 residues away. The remaining residues from the approximately 110-residue C-terminal peptide. The functions of the phosphatase propeptide and C-terminal peptide have not been investigated. The precursor structure of the phosphatase is compared to the precusor structures of other secreted enzymes in Table 3-5.

The probe WT, described in Table 2-4, was prepared by PCR and labelled by random primer synthesis. This probe spans the entire phosphatase gene, rather than a short region. Therefore, any region of *phoA* may be able to bind to homologous DNA sequences from other organisms to search for structurally and enzymatically related enzymes . A number of restriction digests of *L. enzymogenes* and *P. aeruginosa* genomic DNA were separated on an agarose gel, transferred and hybridized with WT (Figure 3-4). Panel B shows that single bands are present in all lanes except for those containing and *P. aeruginosa* DNA. The result indicates that the cloned gene corresponds to a single, uninterrupted DNA sequence in *L. enzymogenes* and suggests that *P. aeruginosa* is not likely to possess any protein similar to the *Lysobacter* phosphatase. However, the hybridization conditions may have been too stringent for locating weak homologies.

3.2.4 Codon usage and %G+C analysis

The nucleotide sequence was analyzed for open reading frames, restriction endonuclease cleavage sites and amino acid codon usage by the method developed by Marck (1988). Table 3-6 compares the codons utilized by *Pseudomonas* spp. *L. enzymogenes* and *E. coli*. The codon usage in *L. enzymogenes* is biased and nearly identical to that of *P. aeruginosa* , which has a similarly high G+C content. Four of the sixty-one sense codons, AUA, ACU, UUA and UUU, are not used by *L. enzymogenes* and seventeen occur less than 6% of the time. The degenerate codons are almost exclusively restricted to G or C in the third position. In contrast, the codon usage is quite

Table 3-5: Structural comparisons of the secreted phosphatase, protease I, and aqualysin I precursor molecules with other known precursors[a].

enzyme (total # residues)	**signal sequence**	**N-terminal propeptide**	**mature enzyme**	**C-terminal extension**
L. enzymogenes phosphatase (539)[1]	29	119	281	110
A. lyticus protease I (653)[2]	20 or 27	185 or 178	268	180
T. aquaticus aqualysin I (513)[3]	19	108	281	105
N. gonorrhoeae IgA protease (1 532)[4]	27	-----	1094[b]	411[b]
S. marcescens protease (1 045)[5]	27	-----	381	637
L. enzymogenes α-lytic protease (396)[6]	24	174	198	-----
P. aeruginosa elastase (498)[7]	≈48	≈150	301	-----
B. subtilis subtilisin E (381)[8]	29	77	275	-----

[a] the number of amino acid residues in each domain is indicated.

[b] based on processing at the furthest autoproteolytic cleavage site.

[1]-this study.

[2]-Ohara, *et al.*, 1989.

[3]-Terada, *et al.*, 1990.

[4]-Pohlner, *et al.*, 1987.

[5]-Yanagida, *et al.*, 1986.

[6]-Epstein and Wensink, 1987.

[7]-Bever and Iglewski, 1988.

[8]-Ohta, *et al.*, 1991.

Figure 3-4: Hybridization of the *phoA* gene probe to *L. enzymogenes* genomic DNA. A. Ethidium bromide-stained agarose gel. B. Autoradiograph of the corresponding Southern transfer. Lanes 1-4 are *L. enzymogenes* digests using *Bgl*II, *Eco*RI, *Eco*RV, and *Hind*III, respectively. Lane 5 is *P. aeruginosa* PAO DNA cut with with *Eco*RI. Lanes 6 and 7 are the molelular weight markers λ*Cla* and λ*Xho*, respectively.

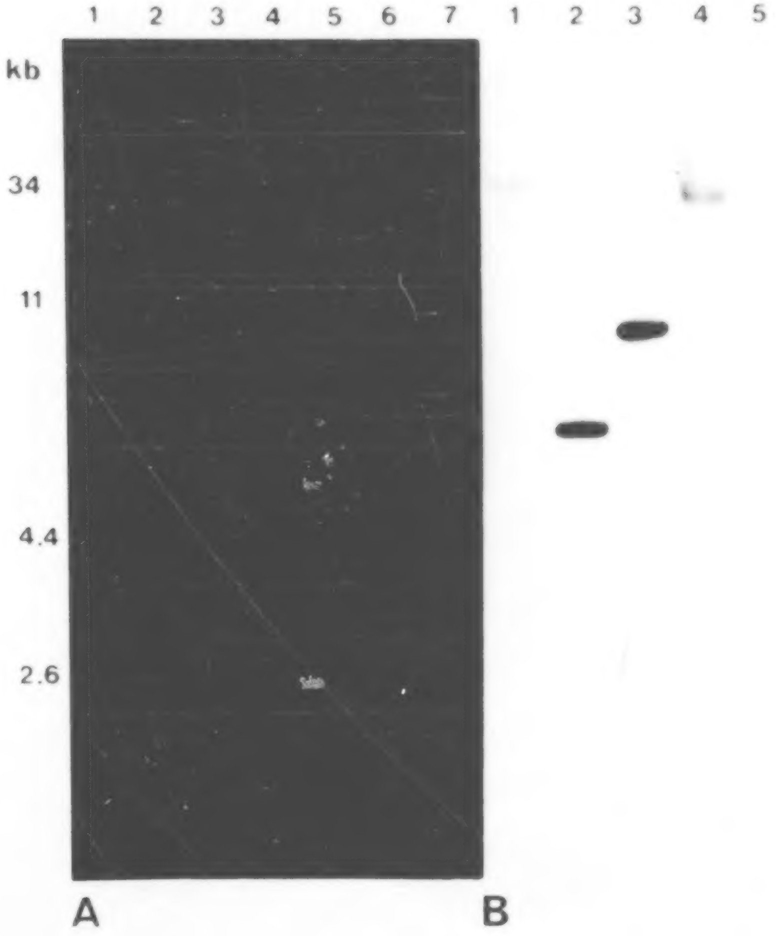

1
2
3
4
5
6
7
1
2
3
4
5
kb
34
11
4.4
2.6
A
B

Table 3-6: Comparison of Codon Usage* between *E. coli*[a] *P. aeruginosa*[b] and *L. enzymogenes*[c].

a.a.	codon	*E.coli*	*P.aer.*	*L.enz.*	a.a.	codon	*E.coli*	*P.aer.*	*L.enz.*
gly	GGG	0.08	0.06	0.05	trp	UGG	1.00	1.00	1.00
“	GGA	0.04	0.04	0.02	OPA	UGA	0.17	0.80	1.00
“	GGU	0.48	0.11	0.05	cys	UGU	0.43	0.05	0.10
“	GGC	0.40	0.79	0.88	“	UGC	0.57	0.95	0.90
glu	GAG	0.27	0.62	0.48	AMB	UAG	0.08	0.07	0.00
“	GAA	0.73	0.38	0.52	OCH	UAA	0.75	0.13	0.00
asp	GAU	0.46	0.14	0.19	tyr	UAU	0.40	0.16	0.09
“	GAC	0.54	0.86	0.81	“	UAC	0.60	0.84	0.91
val	GUG	0.27	0.42	0.52	leu	UUG	0.09	0.09	0.06
“	GUA	0.22	0.07	0.01	“	UUA	0.07	0.01	0.00
“	GUU	0.36	0.04	0.03	phe	UUU	0.37	0.03	0.00
“	GUC	0.15	0.47	0.44	“	UUC	0.63	0.97	1.00
ala	GCG	0.31	0.31	0.40	ser	UCG	0.12	0.27	0.26
“	GCA	0.22	0.03	0.06	“	UCA	0.07	0.01	0.01
“	GCU	0.26	0.07	0.04	“	UCU	0.24	0.01	0.01
“	GCC	0.21	0.59	0.51	“	UCC	0.27	0.28	0.11
arg	AGG	0.01	0.03	0.01	arg	CGG	0.03	0.14	0.11
“	AGA	0.01	0.01	0.01	“	CGA	0.03	0.03	0.08
ser	AGU	0.06	0.04	0.04	“	CGU	0.56	0.10	0.05
“	AGC	0.26	0.40	0.57	“	CGC	0.35	0.68	0.75
lys	AAG	0.24	0.89	0.95	gln	CAG	0.76	0.86	0.81
“	AAA	0.76	0.11	0.05	“	CAA	0.24	0.14	0.19
asn	AAU	0.26	0.08	0.10	his	CAU	0.37	0.22	0.25
“	AAC	0.74	0.92	0.90	“	CAC	0.63	0.78	0.75
met	AUG	1.00	1.00	1.00	leu	CUG	0.68	0.64	0.63
ile	AUA	0.03	0.01	0.00	“	CUA	0.02	0.01	0.01
“	AUU	0.36	0.06	0.09	“	CUU	0.07	0.02	0.01
“	AUC	0.61	0.94	0.91	“	CUC	0.07	0.24	0.28
thr	ACG	0.17	0.13	0.17	pro	CCG	0.65	0.62	0.63
“	ACA	0.07	0.02	0.01	“	CCA	0.16	0.03	0.02
“	ACU	0.25	0.03	0.00	“	CCU	0.12	0.03	0.07
“	ACC	0.51	0.83	0.82	“	CCC	0.07	0.32	0.28

* fraction of specific codon occurences per # total codons in synonymous group.

[a] *E. coli*. 52 genes and 16351 codons analyzed (Alff-Steinberger, 1984).

[b] *P. aeruginosa*. 15 genes and 5663 codons analyzed (West and Iglewski, 1988).

[c] *L. enzymogenes*. 3 genes and 1241 codons analyzed.(Epstein and Wensink, 1987; Boras, *et al.*, 1993; this study).

different from that of *E. coli* where the third position of ala and val codons have G, A, T or C with about equal frequencies while other codons such as for gly, arg, thr and ile favor pyrimidines over purines. Of the amino acids which are represented by only two codons, Cs and As are generally preferred to Us and Gs, respectively (Alff-Steinberger, 1984).

The %G+C content for each of the three codon positions of *phoA* was determined by a %G+C analysis program (Bibb, *et al.*, 1984) as modified for the Macintosh™ computer by Dr. S.E. Jensen (Figure 3-5). The arrow shows the position of *phoA* which aligns closely with the region showing the open appearance which typifies the open reading frame (phase 3). Through this region, there are almost exclusively Gs or Cs in the third codon position. In the regions immediately flanking the gene, base selection is essentially random in all phases. These results support the boundaries of the open reading frame described in Figure 3-2.

3.2.5 Homologies to other known proteins

Amino acid sequence homology searches revealed that the secreted phosphatase of *L. enzymogenes* shows significant similarities to purple, iron-containing acid phosphatases from mammalian sources such as porcine uterus, bovine spleen, human macrophage, placenta, bone and spleen (Ketcham, *et al.*, 1985; Hunt, *et al.*, 1987; Hayman, *et al.*, 1989; Ketcham, *et al.*, 1989; Lord, *et al.*, 1990). The region of the highest homology between the enzymes of *L. enzymogenes* and two acid phosphatases from bovine spleen and human macrophage are shown in Figure 3-6. The sequences 'WLK', 'LAA' and 'GHDHN' are common to these proteins. The other mammalian acid phosphatases have similarly conserved residues. There is an identity of greater than 30% through a 70 amino acid overlapping region. The approximately twenty protein sequences obtained by the homology search did not identify any other phosphatases . The visible spectrum of the purified *L. enzymogenes* enzyme exhibited a maximum absorption at 525 nm which is

Figure 3-5: % G+C codon analysis of *phoA*. The nucleotide sequence shown in figure 3-2 was used in the determination of %G+C described by Bibb *et al.* (1984). Each line represents the average %G+C content as a fraction of position within the codon using a window of 40 codons for phase 1 (line 1), phase 2 (line 2), and phase 3 (line 3). The arrow indicates the open reading frame that encodes the secreted phosphatase.

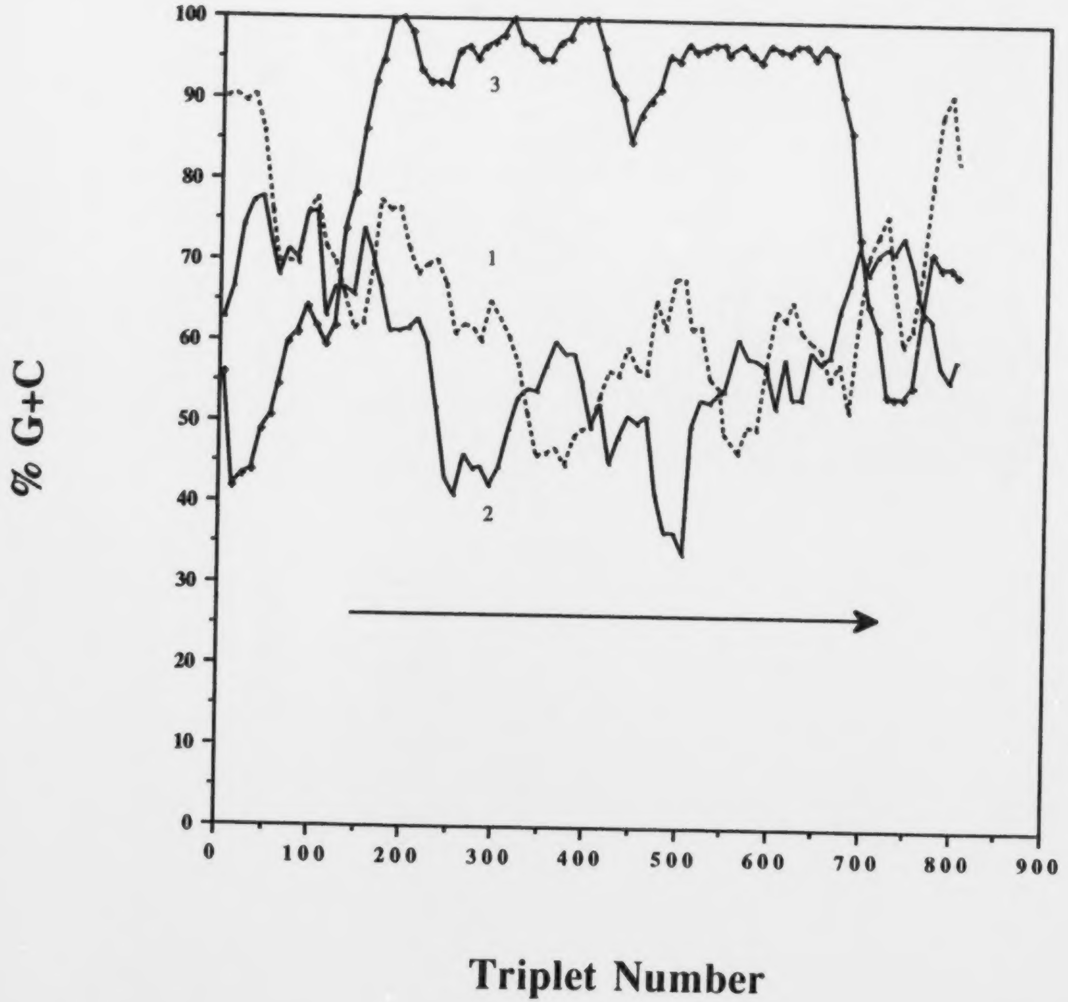

% G+C
100
90
80
70
60
50
40
30
20
10
0
100
200
300
400
500
600
700
800
900
3
1
2
Triplet Number

Figure 3-6: Region of greatest amino acid homology between the *Lysobacter* phosphatase, bovine spleen and human macrophage acid phosphatases. Amino acid residues are numbered as in Figure 3-2. Vertical lines indicate the exact matches. Conservative substitutions are not indicated. A hyphen is used for spacing to maintain alignment and the 'X' in the bovine spleen acid phosphatase sequence may be Ile or Leu.

```
residue #           110                           140
L. enzymogenes      DWHFVSLNTMSGGTVAQAQIDWLKADLAANTKPCTAAYFHHPLLSR
                              |   ||  |  |||  |||          | |  |
bovine spleen       MXDTVTXCGNSDDFVARTQLAWLKKQLAAAKEDYVLVAGHYPVWSX
                                   |  |  |||  |||          | |  |
human macrophage    DFLSQQPERPRDVKLARTQLSWLKKQLAAAREDYVLVAGHYPVWSI

                                  170                            199
L. enzymogenes      GSYS-GYSQVKPFWDALYAAKADLVLVGHDHNYQRYGKMNPDKAAA
                             ||     | | |    | ||||| | |
bovine spleen.      AEHGVVHCXVKQXXPXXNAHKVTAYXCGHDHNXQ-YXQQENGXFGV
                             ||     |        | ||||| | |
human macrophage.   AEHGPTHCLVKQLRPLLATYGVTAYLCGHDHNLQLYQ-DENGVGYV
```

similar to the mammalian enzymes. Several iron-chelators were used to determine whether they have any inhibitory effect on the phosphatase. None were able to inhibit enzyme activity after pre-incubation with the phosphatase for 1 h at 37°C (not shown), which could suggests that the iron is inaccessible or very tightly associated with the protein.

3.2.6 Detection of iron by atomic absorption

The purified phosphatase was redissolved to 1 mg/mL based on Bio-Rad protein determinations, which corresponds to about 33 nmol protein/mL assuming a molecular weight of approximately 30 kDa. Results from the atomic absorption analysis showed that there was approximately 15.3 nmol/mL of Fe in the protein sample. This indicates that iron was present at an iron to protein ratio of about 0.46:1. Thus, it can be assumed that the protein contains one mole of Fe per mole of protein. The low iron value may be due to loss of Fe during preparation, impurities in the sample and/or inaccuracies in the protein determinations.

3.3 Expression of the secreted phosphatase in *E. coli*

3.3.1 Preparation of recombinant expression plasmids

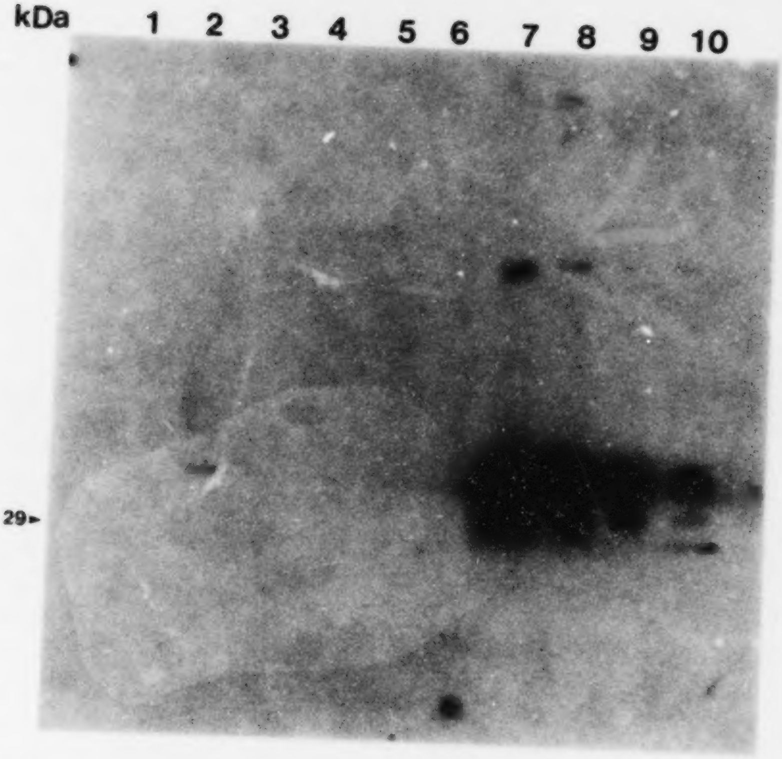
Custom oligonucleotide primers (Table 2-3) were used in PCR reactions to prepare DNA fragments that contain the alkaline phosphatase gene, cloning sites and the consensus ribosome-binding site (Table 2-4). The PCR product M3 was used in most of the cloning and expression experiments. M3 contains the entire *phoA* gene with a GTG to ATG modification of the initiator codon, an alteration of the Shine-Dalgarno sequence and the addition of three restriction sites, *Eco*RI, *Nde*I and *Xho*I.

M3 was cut with *Eco*RI-*Xho*I and cloned into pKK223-3 and pUC118, which were each cut with *Eco*RI-*Sal*I. Isolates were screened by colony hybridization and positive colonies were retained and the plasmids were called pKKM3 and pESM3, respectively. pUC118 was chosen because it was successful in expressing the β-lactamase from *L. enzymogenes* (Boras, *et al.*, 1993). M3 and pBluescriptIIKS$^+$ were each cut with *Eco*RI-*Xho*I, ligated and used to transform *E. coli* MV1193. M3 was also digested with *Nde*I-*Xho*I and ligated into pT7-7 which had been digested with *Nde*I-*Sal*I. These recombinants were called pKSM3 and pT77M3, respectively. The recombinant plasmids were isolated and used to transform *E. coli* JM105 (DE3) or *E. coli* K38/pGP1-2), both of which contain an inducible T7-RNA polymerase. The cloning regions of all recombinant vectors were sequenced to confirm the identities of the vectors and the inserts.

3.3.2 Western transfer and immunodetection

Protein samples which might have contained the recombinant phosphatase were separated by SDS-PAGE and transferred to nylon membranes in preparation for immunological detection experiments. It was found that the presence of β-mercaptoethanol and subsequent heating reduced the amount of enzyme that could be detected by at least one hundred-fold (Figure 3-7). Moreover, prolonged heating of the samples prior to SDS-PAGE in the absence of β-mercaptoethanol also reduced the sensitivity of detection of the enzyme (Figure 3-8). The heat treatment caused limited denaturation of the phosphatase as indicated by the shift in mobility of the protein and the epitope recognized by anti-AP remained intact for a short period of time. Therefore, subsequent samples were heated for a maximum of 1 min at 95°C without the use of β-mercaptoethanol. The conditions for the Western transfer and for the detection of the secreted phosphatase were optimized using purified enzyme as a positive control. Figure 3-9 is an immunoblot which contains proteins from alkaline phosphatase purification samples. The sensitivity of detection was

Figure 3-7: Immunological analysis of β-mercaptoethanol/heat-denaturated secreted phosphatase. Various amounts of purified phosphatase were separated by SDS-PAGE, electrotransferred onto a nylon membrane and immunoblotted with α-AP. Lane 1 contains the molecular weight standards phosphorylase b (96 kDa), BSA (67 kDa), OVA (46 kDa), carbonic anhydrase (29 kDa), chymotrypsinogen (25 kDa), lysozyme (14 kDa), and cytochrome C (12 kDa). Lanes 2-6 contain 4 μg, 1.2 μg, 0.4 μg, 0.12 μg and 0.04 μg of purified enzyme treated with β-mercaptoethanol and 10 minutes of heating at 95°C. Lanes 7-10 contain 4 μg, 1.2 μg, 0.4 μg, and 0.12 μg of purified enzyme without β-mercaptoethanol and not heated.


kDa
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10
29

Figure 3-8: Immunological analysis of heat-denaturated secreted phosphatase. *L. enzymogenes* cultures were grown in 0.8% soy peptone for 18 hr and 100 μL aliquots of culture supernatant were removed and heated at 95°C for 0, 1.5, 5, 7, 10, 15 and 20 min without β-mercaptoethanol prior to SDS-PAGE and electrotransfer.

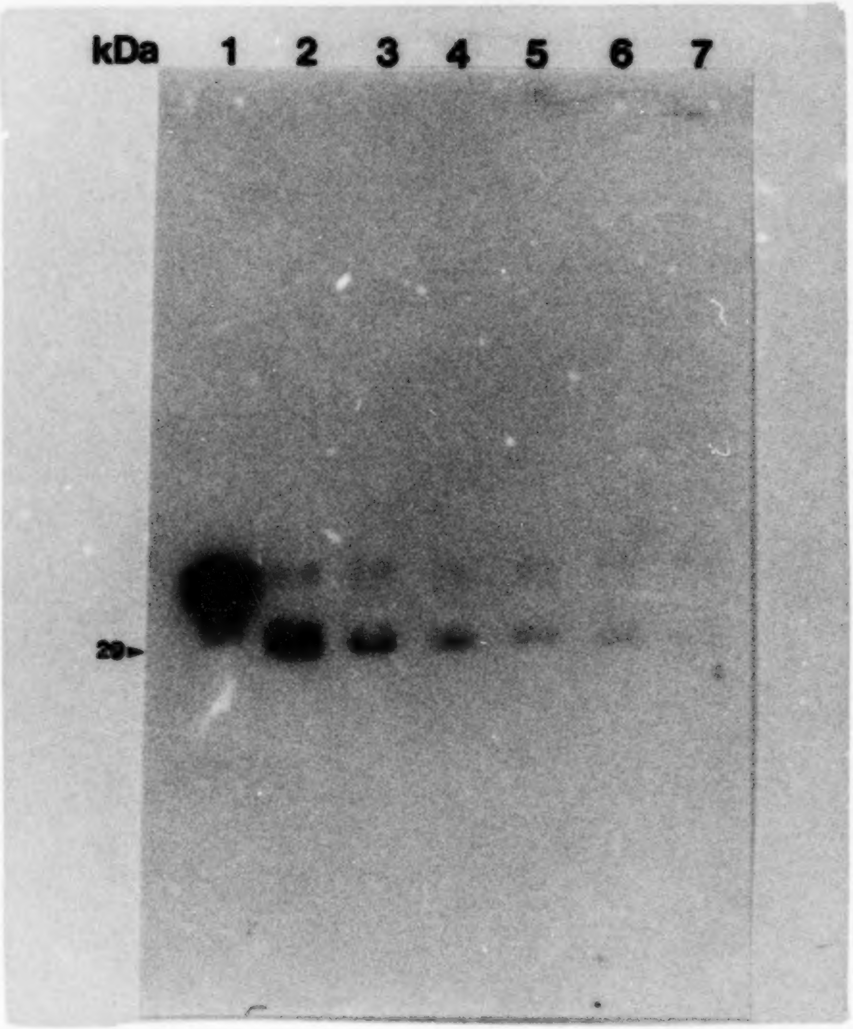
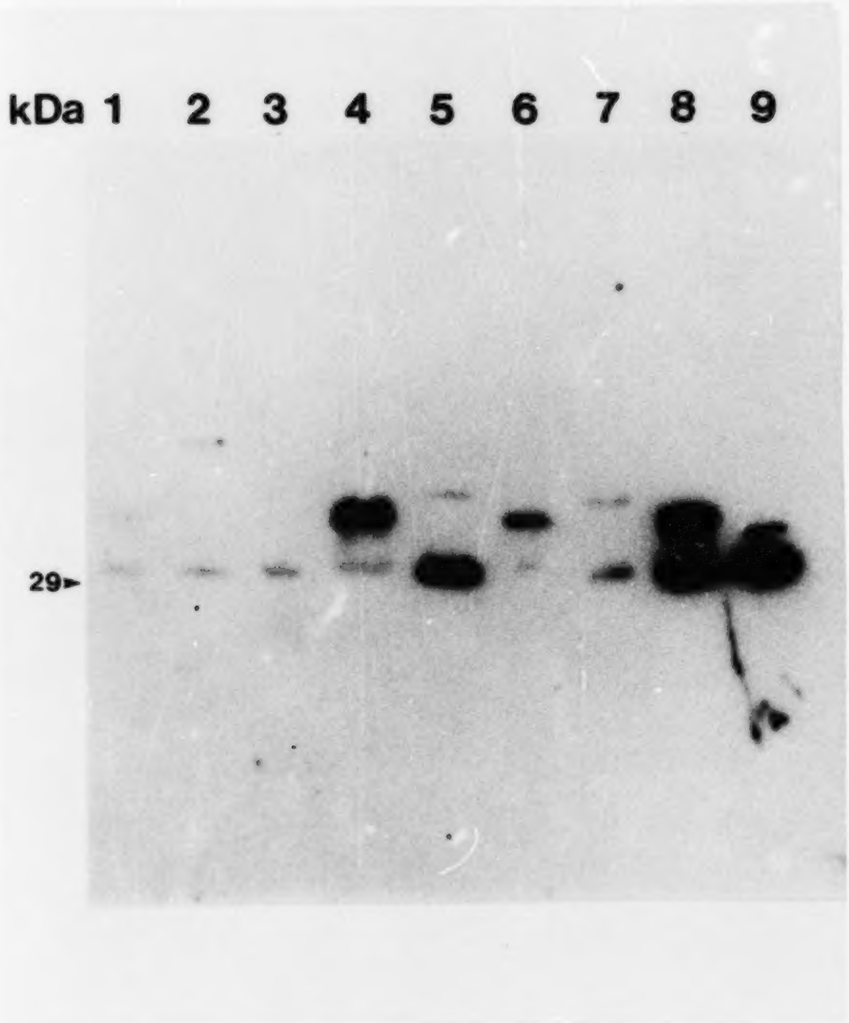

kDa
1
2
3
4
5
6
7
29

Figure 3-9: Immunological analysis of *L. enzymogenes* secreted phosphatase purification samples. Lane 1 contains 0.1 µg phosphatase which was not heated. Lane 2 has 75 µL whole cells that was heated for 1 min at 95°C. Lane 3 has heated acetone-precipitated proteins from 100 µL of culture supernatant. Lanes 4, 6 and 8 contain 25 µL CM-52 cellulose-concentrated enzyme, 0.1 µg purified phosphatase with 6.6 M urea, and 1.0 µg purified phosphatase that were not heated. Lanes 5, 7 and 9 contain the same samples, but heated.


kDa
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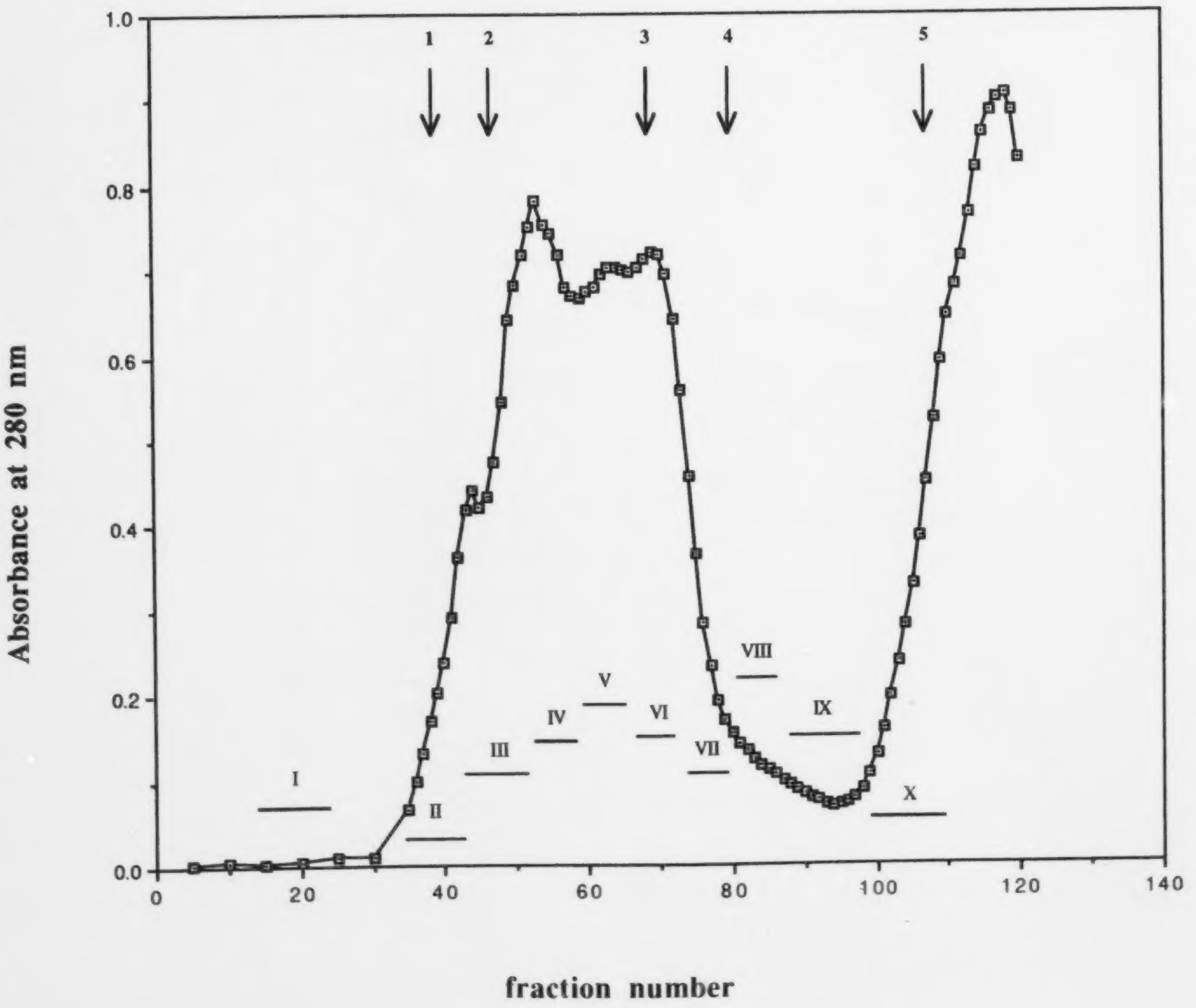
8
9
29►

significantly better, but the protein bands do not necessarily correspond to their true molecular weights. The appearance of multiple bands from samples of purified enzyme on the autoradiographs probably is due to the partial denaturation of the native enzyme during the SDS/heat treatment. The presence of 6.6 M urea in addition to SDS in the gel loading buffer did not appear to affect detection of the protein (Figure 3-9, lanes 6 and 7).

3.3.3 Expression with *tac* and *lac* vectors

Shock fluid from each culture of *E. coli* MV1193 transformed with the appropriate recombinant plasmid was isolated and concentrated as described in Materials and Methods, Section 2.6.3. The concentrated shock fluid was separated on a Sephadex G-75 Superfine column and fractions were pooled as indicated in Table 2-5 and Figure 3-10. The pooled fractions were assayed for β-lactamase and phosphatase activity, and those with significant phosphatase activity were incubated with EDTA and with anti-AP in order to differentiate between the recombinant phosphatase and the host phosphatase. Table 3-7 shows results from one of the experiments. The amount of active phosphatase can vary by up to 50% in different experiments. As a control, purified phosphatase from *L. enzymogenes* was shown to be inhibited by anti-AP but not by EDTA. The Sephadex pools IV-VI from *E. coli* MV1193/pES10 had a significant amount of EDTA-resistant and anti-AP-sensitive activities, and activity was highest in pool V. Sephadex pool III seemed to have a mixture of both the host and recombinant phosphatases, which is expected since the *E. coli* phosphatase has a higher molecular weight and should elute earlier than the *L. enzymogenes* enzyme. Most of the β-lactamase, which has a molecular weight of 28 961 Da (Sutcliffe, 1978), was contained in pool VI (not shown). As expected, the Sephadex pools from *E. coli* MV1193/pUC118 contained only EDTA-sensitive and anti-AP-resistant activities.

Figure 3-10: A gel filtration profile of concentrated shock fluid on Sephadex G-75 Superfine column. The proteins were eluted from the column and 2 mL fractions were collected. The arrows 1 through 5 indicate the elution points of blue dextran (V_0), OVA (46 kDa), secreted alkaline phosphatase (30 kDa), cytochrome C (12 kDa), and NH_4^+ (V_t). The horizontal lines labelled I through X represent the pooled Sephadex fractions as prepared according to Table 2-5.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5
I
II
III
IV
V
VI
VII
VIII
IX
X
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
140
Absorbance at 280 nm
fraction number

Table 3-7: Enzyme assays of concentrated shock fluid and pooled Sephadex fractions.

sample	enzyme activity (milliunits per ml of culture)	% inhibition by 5 mM EDTA	% inhibition by anti-AP (1:5)
***E. coli* MV1193/pES10 + IPTG**			
conc. shock fluid	6.2	1%	92.4%
Seph. I	0.0	-----	-----
Seph. II	0.0	-----	-----
Seph. III	0.65	26%	81%
Seph. IV	6.6	27%	55%
Seph. V	9.15	<1%	97%
Seph. VI	2.15	4%	99%
Seph. VII	0.0	-----	-----
Seph. VIII	0.0	-----	-----
Total Seph.	18.55		
***E. coli* MV1193/pUC118**			
conc. shock fluid	0.49	58%	1.1%
Seph. I	0.0	-----	-----
Seph. II	0.0	-----	-----
Seph. III	0.47	75%	<1%
Seph. IV	0.04	-----	-----
Seph. V	0.0	-----	-----
Seph. VI	0.01	-----	-----
Seph. VII	0.0	-----	-----
Seph. VIII	0.01	-----	-----
Total Seph.	0.53		
L. enzymogenes			
supernatant	780	<1%	99%
purified phosphatase		<1%	99%

The culture supernatant of *E. coli* MV1193/pES10 was also assayed for phosphatase activity. Approximately two-thirds of the total active phosphatase was in the supernatant and one-third was in the shock fluid (not shown). Furthermore, significant amounts of β-lactamase were also detected in the *E. coli* MV1193/pUC118 culture supernatants and shock fluids, which is in agreement with previous observations (Boras, *et al.*, 1993).

The enzyme activity obtained from *L. enzymogenes* culture supernatant was usually around 780 milliunits/mL, whereas only 6.2 milliunits/mL were detected in the shock fluid from a culture of *E. coli* MV1193/pES10. However, the combined activity of the Sephadex pools from the *E. coli*/pES10 concentrated shock fluid totalled 18.7 enzyme milliunits/mL after gel filtration, which is an increase of about 3-fold. A 3- to 6-fold increase of phosphatase activity was observed in several expression experiments. However, this increase was not seen when concentrated shock fluid was dialyzed or incubated at 0°C. There was no significant change in the amount of β-lactamase detected in the shock fluid, concentrated shock fluid or the pooled Sephadex fractions. It appears then that there may be some enzyme activation, possibly by processing of the precursor and/or removal of an inhibitor which occurs only during the gel filtration procedure.

Sephadex pools I through VIII from both cultures were analyzed by SDS-PAGE and immunoblots. The stained gel of the samples from *E. coli* MV1193/pUC118 is shown in Figure 3-11. There was no binding of anti-AP to proteins from any *E. coli* MV1193/pUC118 Sephadex pools after Western transfer (not shown). *E. coli* MV1193/pES10 stained gel and immunoblots are shown in Figures 3-12, 3-13 and 3-14. Sephadex pool V, which has the highest enzyme activity of all Sephadex pools, exhibits the strongest signal on the immunoblot . A total of 0.08 units of enzyme from pool V (Figure 3-13, lane 6) and 0.015 units of purified phosphatase (Figure 3-13, lane 10) were used in the immunoblot. The differences in enzyme content are consistent with differences in the band intensities. Higher molecular weight species, which may be phosphatase

Figure 3-11: Coomassie Blue-stained SDS-polyacrylamide gel of shock fluid and Sephadex samples from *E. coli*/pUC118. Lane 1 has molecular weight standards phosphorylase b (96 kDa), BSA (67 kDa), OVA (46 kDa), carbonic anhydrase (29 kDa), chymotrypsinogen (25 kDa), lysozyme (14 kDa), and cytochrome C (12 kDa). Lanes 2-10 contain 20 μL each of concentrated shock fluid and Sephadex fractions I-VIII. All samples were heated for 1 min at 95°C and did not contain β-mercaptoethanol.

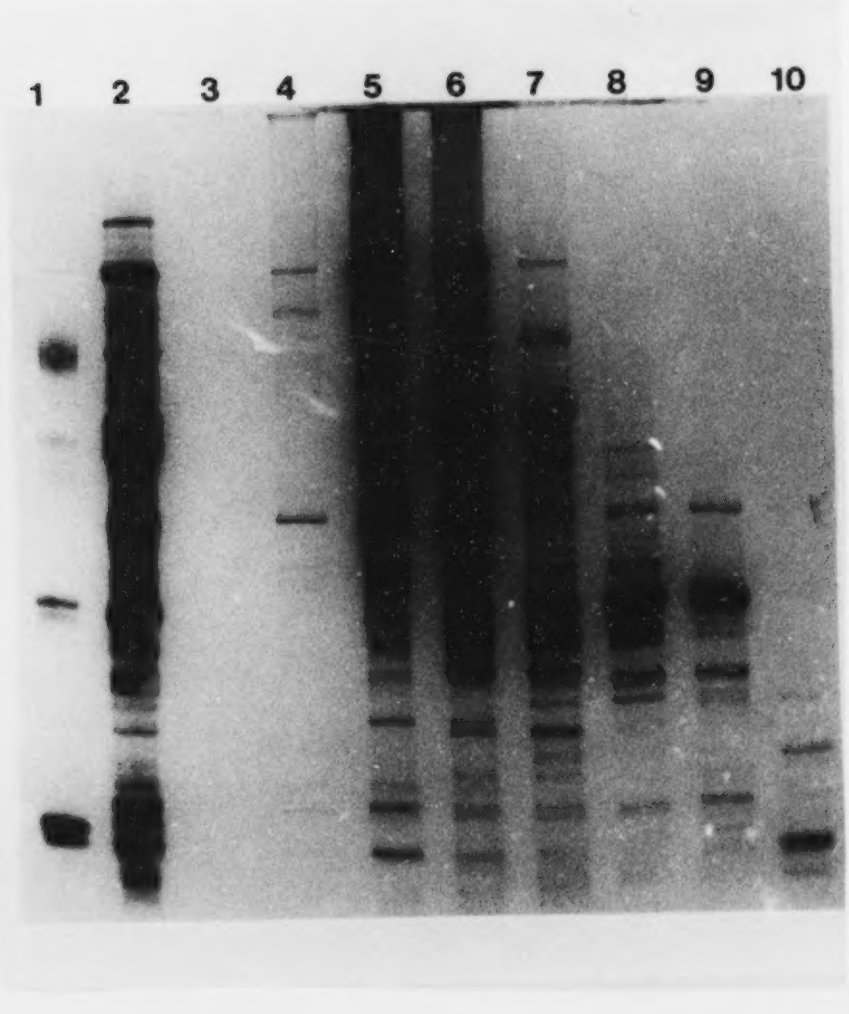
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Figure 3-12: Coomassie Blue-stained SDS-polyacrylamide gel of shock fluid and Sephadex samples from *E. coli*/pES10. Lane 1 has molecular weight standards phosphorylase b (96 kDa), BSA (67 kDa), OVA (46 kDa), carbonic anhydrase (29 kDa), chymotrypsinogen (25 kDa), lysozyme (14 kDa), and cytochrome C (12 kDa). Lanes 2-10 contain 20 µL each of concentrated shock fluid and Sephadex fractions I-VIII. All samples were heated for 1 min at 95°C and did not contain β-mercaptoethanol.

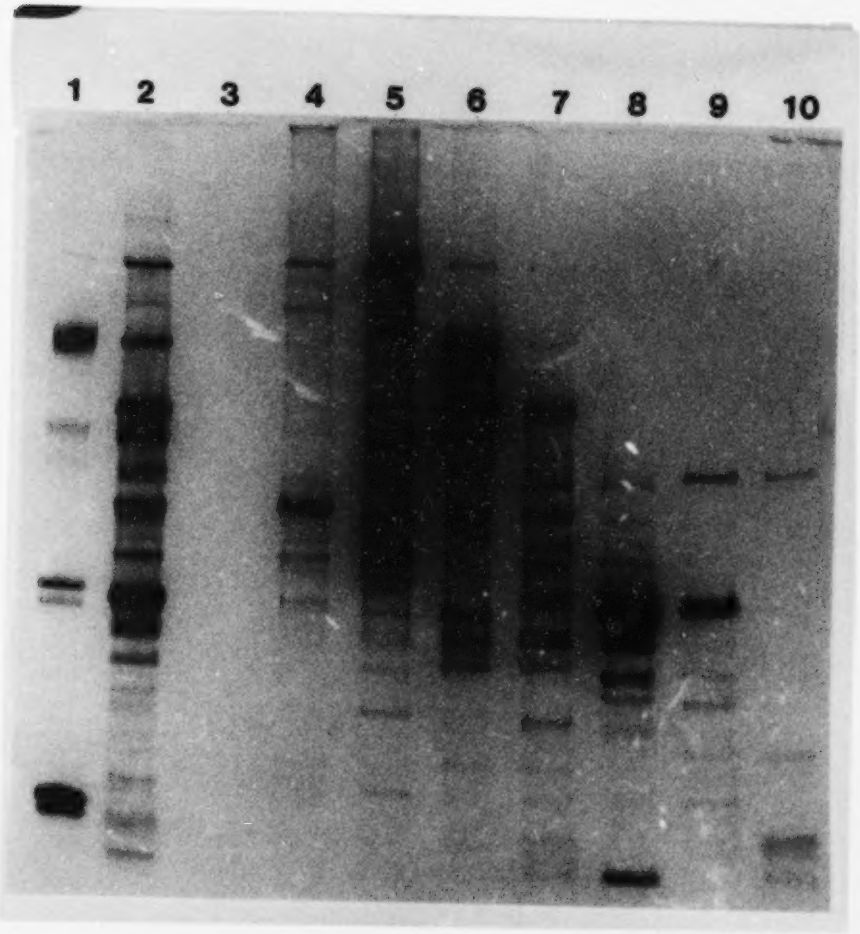
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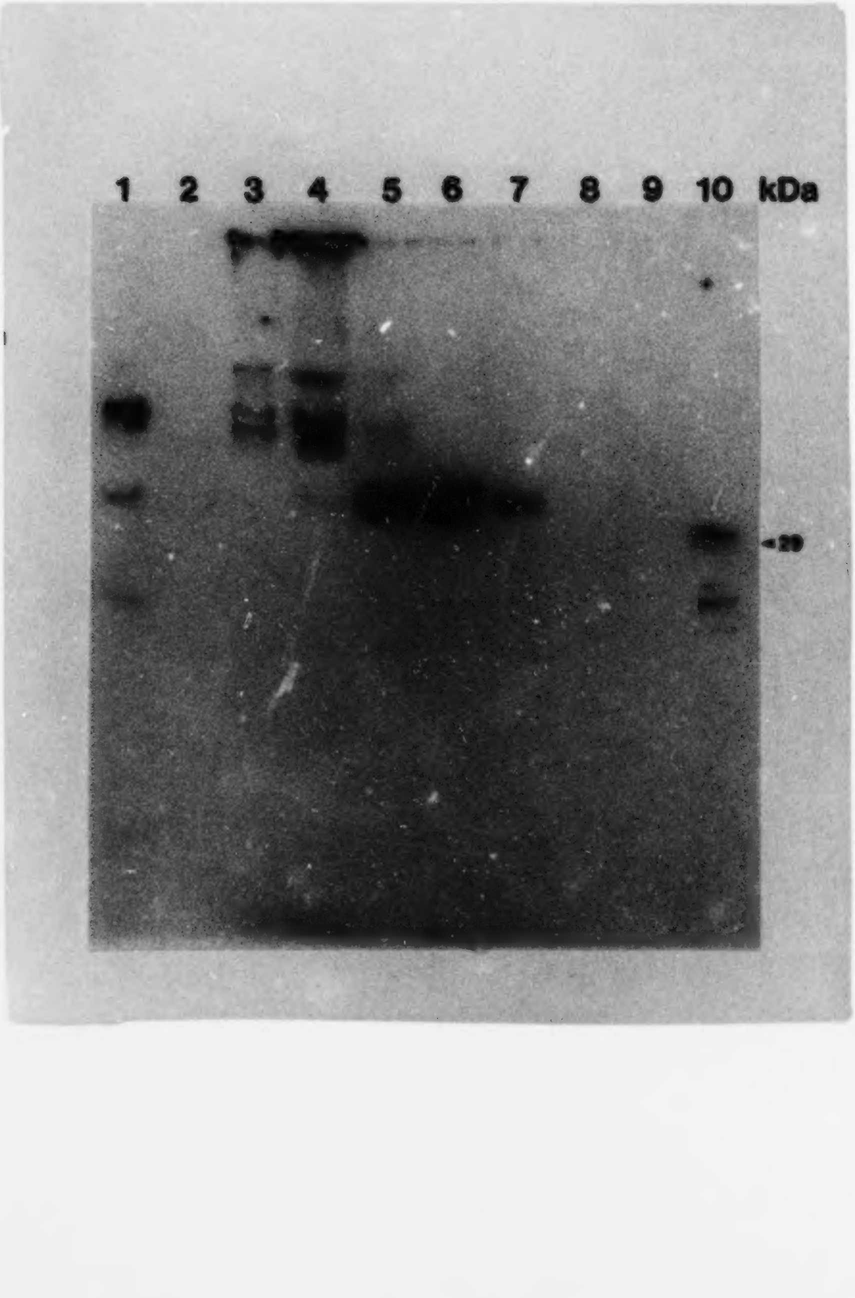
Figure 3-13: Western transfer and immunoblot of shock fluid and pooled Sephadex samples from *E. coli*/pES10. Lane 1 has 20 μl of concentrated shock fluid. Lanes 2-9 contain 40 μL each of Sephadex fractions I-VIII. Lane 10 has 0.1 μg heated purified phosphatase. All samples did not contain β-mercaptoethanol and were not heated.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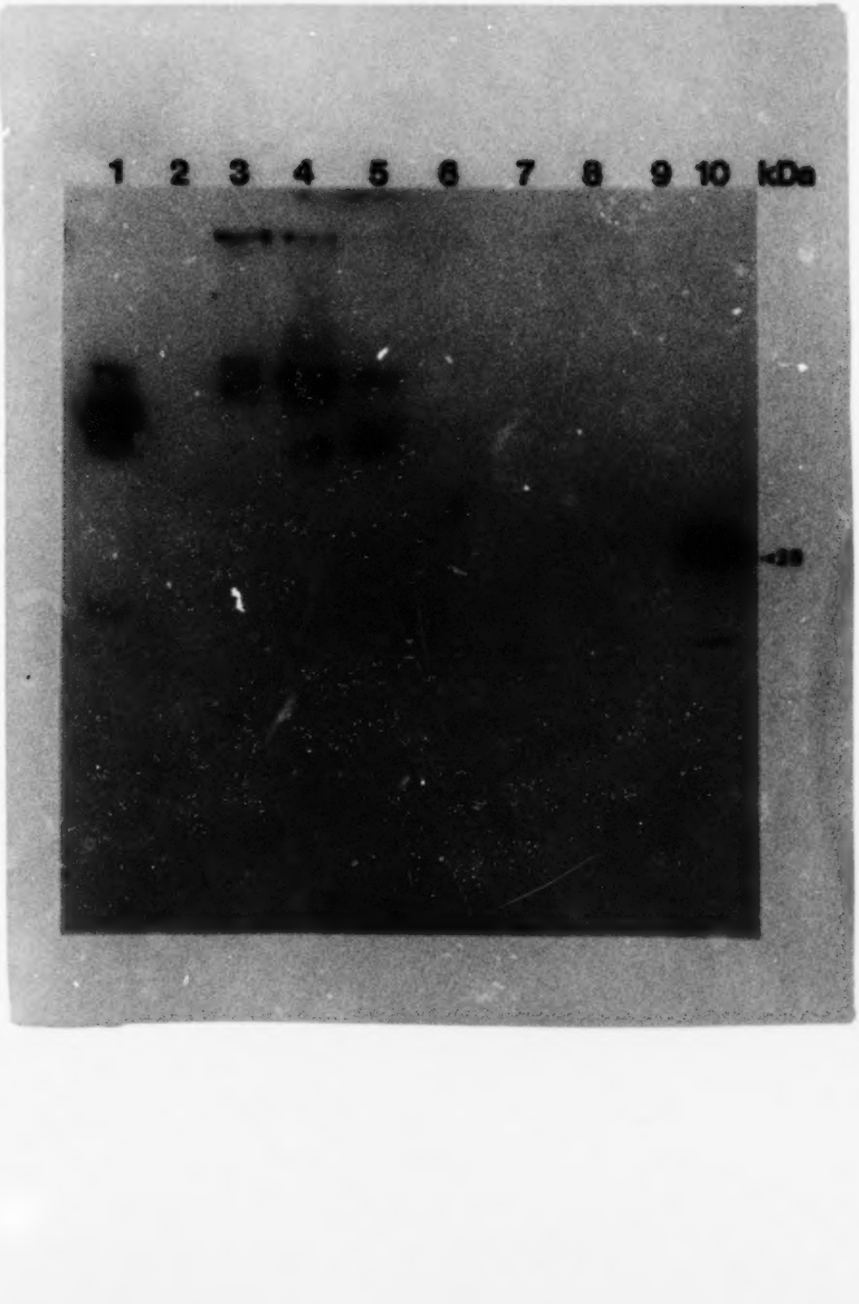
kDa
◄29

Figure 3-14: Western transfer and immunoblot of heat-denatured shock fluid and pooled Sephadex samples from *E. coli*/pES10. Lane 1 has 20 μl of concentrated shock fluid. Lanes 2-9 contain 40 μL each of Sephadex fractions I-VIII. Lane 10 has 0.1 μg heated purified phosphatase. All samples were heated at 95°C for 1 min and did not contain β-mercaptoethanol.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intermediates, were recognized by anti-AP (lanes 3-5). Furthermore, the approximately 30 kDa band corresponding to the mature phosphatase was heat-labile since it disappeared after heating (Figure 3-14, lanes 1, 5-7). This is in agreement with the results using purified phosphatase.

The same volume of concentrated shock fluid from *E. coli*/pES10 was also applied onto a 75 mL CM-52 cellulose column in an attempt to partially purify the enzyme by a different method. However, when the pH of the concentrated shock fluid was adjusted to 5.0 with acetic acid, a large amount of protein precipitated out of solution and resulted in a loss of at least 50% of the anti-AP-sensitive activity present in the sample. After ion exchange, only about 25% of the enzyme activity was recovered. There was no appreciable increase in the amount of phosphatase activity in *E. coli*/pUC118 concentrated shock fluid before or after ion exchange. The low pH may have caused the precipitation of the precursor molecules from solution or altered the conditions such that activation could not occur.

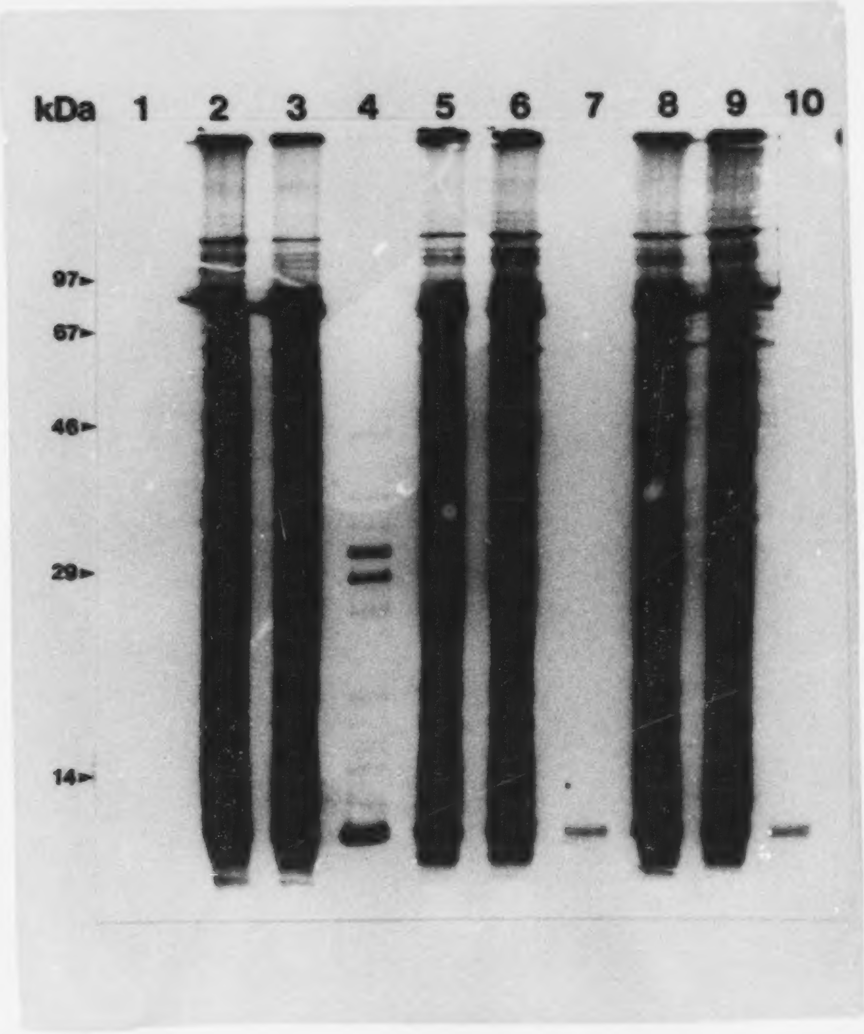
Other constructs were also prepared in order to express truncated forms of the phosphatase. The PCR product -SD (Table 2-4) was cloned into pTTQ18 and called pTTQM3. It was only used in experiments to prepare concentrated shock fluid and it was not examined any further since no enzyme activity was detected. PCR products Mat and PP were used to clone into pUC118. Sequencing of these recombinant vectors showed that there was at least one single-base deletion within the first fifty nucleotides of the cloning region. In one case, there was a two-base deletion. The same PCR products were also cloned into the expression vector pINompA1. Expression experiments using these clones were not successful.

3.3.4 Expression from T7 vectors

Expression experiments with T7 vectors were used first in an attempt to detect the production of recombinant enzyme by *in vivo*-labelling of proteins. The T7 promotors in pT7-7 and pBluescriptIIKS$^+$ allowed the selective transcription of the recombinant gene. The addition of rifampicin inhibits host RNA polymerase and results in reduced production of host proteins, but not T7-controlled plasmid-encoded proteins. Cells with the appropriate plasmid were grown and the samples were prepared as described in Materials and Methods, Section 2.6.2. pT7-3 was used as an internal positive control since the β-lactamase gene is oriented in the same direction as the T7-promotor. The presence of a band in the rifampicin-containing samples on an autoradiograph would show that the T7 RNA-polymerase was active. The results from the ^{35}S-Met labelling experiment are shown in Figure 3-15. The positive control demonstrates the production of two heavy bands at about 32 kDa and 30 kDa by pT7-3 in the presence of rifampicin (lane 4) which seems to correspond to the β-lactamase precursor and mature enzyme, respectively. There are no unique bands produced in the rifampicin-containing samples of pT77M3 and pKSM3 (lanes 7 and 10). Experiments were repeated using *E. coli* K38/pGP1-2 transformed with the same vectors. The autoradiograph showed that phosphatase-like protein was not synthesized (not shown).

The gene for the *L. enzymogenes* secreted phosphatase has been identified and characterized and several interesting features of the phosphatase have been observed. Its four-domain precursor structure has not been seen in other extracellular proteins, except for some proteases. The homology to mammalian acid phosphatases and the presence of iron indicate a distant evolutionary link in the development of these enzymes. Although the amount of active phosphatase produced by *E. coli* /pES10 is small, the results undoubtedly demonstrate that *phoA* was expressed and that at least some of the precursor was converted to the active form. The consistent increase in enzyme activity from partially purified *E. coli*

Figure 3-15: Expression of the phosphatase gene in *E. coli* using T7 vectors. *E. coli* JM109 (DE3) cells were transformed with the appropriate vector and grown as described. Samples were labelled with ^{35}S-methionine for 5 min and the products separated by SDS-PAGE, dried, and autoradiographed. Lane 1 contains the molecular weight standards phosphorylase b (96 kDa), BSA (67 kDa), OVA (46 kDa), carbonic anhydrase (29 kDa) and lysozyme (14 kDa). Lanes 2-4 contain labelled products of pT7-3 from uninduced cells, induced cells, and induced cells plus rifampicin, respectively. Lanes 5-7 and lanes 8-10 have similar samples from cells containing pT77M3 and pKSM3, respectively.


kDa
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97
67
46
29
14

shock fluid allows the speculation of a role that the N- and C-terminal extensions may play in processing and secretion of the phosphatase.

CHAPTER 4

Discussion

The secreted alkaline phosphatase from *L. enzymogenes* was purified and the information from N-terminal amino acid analysis was used to search for and to isolate its gene, *phoA*. During SDS-PAGE using slab gels, the phosphatase migrated slightly behind one of the protein standards, carbonic anhydrase, which has a molecular weight of 28 980 Da. Thus, the molecular weight of the phosphatase was estimated to be 30 kDa in contrast to a previous estimate of 25 kDa when tube gels were used (von Tigerstrom, 1984). It was expected that a protein of this size would contain no more than 300 amino acid residues and that its precursor would likely possess a signal sequence. Therefore, it was thought that the phosphatase was likely to be encoded by a gene of approximately 1 kb in length. However, a sequence of over 2.5 kb was eventually obtained before the entire structural gene of the phosphatase was elucidated. The sequence was analyzed for open reading frames, codon preferences and G+C distribution. The %G+C analysis shows that the nucleotide sequence through the coding region aligns closely with the region which has mainly Gs or Cs in the third position of the amino acid codons. The identity of the gene was confirmed by comparing the amino acid sequence deduced from the DNA sequence with the N-terminal amino acid sequences obtained for the mature enzyme and the two CNBr fragments. It is clear that the amino acids not identified by N-terminal sequencing at positions 12 and 19 of the mature N-terminus are cysteine residues and the questionable threonine residue at position 28 is a serine.

The results showed that the gene is a unique and continuous DNA sequence coding for a polypeptide that is 539 residues in length with a molecular weight of 56 422 Da, about twice the size of the mature phosphatase. The signal sequence and the mature phosphatase sequence are separated by a 119-residue N-terminal propeptide which has a molecular weight of 13 096 Da. The signal sequence, N-terminal propeptide and the mature enzyme account for almost 80% of the length of the precursor. From this, it is clear that there is also a C-terminal extension of approximately 100 to 120 residues immediately following the mature protein. However, the C-terminal residue of the mature protein and the length of the C-terminal extension are unknown. Unfortunately, C-terminal sequencing experiments did little to identify the C-terminal residue of the mature phosphatase.

The identity of the C-terminal residue was approximated using the information from SDS-PAGE and N-terminal amino acid sequencing information. CNBr fragment #1 contains the region between Asn-194 to the carboxy-terminus of the mature phosphatase and CNBr fragment #2 seems to be the 74-residue, 8 167 Da peptide from Ser-120 to Met-193. The similar electrophoretic mobilities of the two CNBr fragments allowed the estimation of CNBr fragment #1 to be about 81 residues long with a molecular weight of 8 203 Da. This would give a 274-residue mature enzyme with a molecular weight of 28 869 Da. However, if the approximate molecular weight of the mature enzyme is 30 kDa, then Asn-281, which is 88 residues from Asn-194, is also a potential C-terminal residue. This would predict a molecular weight of 29 864 Da for the mature phosphatase. Thus, the molecular weight of the 110-residue C-terminal peptide would be 11 055 Da.

The presence of the N-terminal and C-terminal extensions was unexpected because they are relatively common among extracellular protease precursors, such as the IgA protease and the α-lytic protease, but not among precursors of other extracellular enzymes. It is likely that processing of these domains occurs prior to secretion of the mature enzyme. Since the phosphatase is secreted in conjuction with proteases, limited proteolytic cleavage of the phosphatase at the C-terminal end may be a potential problem for the identification of the C-terminal amino acid using carboxypeptidase. If proteolysis occurs during the purification process, then C-terminal digestion with carboxypeptidase may release residues from non-identical substrates throughout the reaction period.

Other studies have reported secreted enzymes which are synthesized with a four domain precursor structure; the protease I of *A. lyticus* (Ohara, *et al.*, 1989), the aqualysin I of *T. aquaticus*. (Terada, *et al.*, 1990) and the HA/protease and metalloprotease of *Vibrio* spp. (Häse and Finkelstein, 1991; Milton, *et al.*, 1992). The sizes of the respective signal sequences, N-terminal propeptide, mature enzyme, and C-terminal extension are very similar to each other. The aqualysin has been expressed in *E. coli* (Terada, *et al.*, 1990) where it remained associated in the inactive form with the outer membrane fraction and was activated by heating at 65°C. In a related strain, *T. thermophilus* (Touhara, *et al.*, 1991), the protease is secreted and autocatalytically activated, although much more slowly than in *T. aquaticus*. It has been suggested that there is no requirement for a specific secretion apparatus for aqualysin I and that the targeting information lies somewhere in the precursor structure (Touhara, *et al.*, 1991). Similarly, the *Achromobacter* protease is produced in *E. coli*, but not transported through the outer membrane (Ohara, *et al.*, 1989). Thus, the N- and C-terminal peptides do not enable aqualysin nor protease I to be secreted, at least not in *E. coli*. *E. coli* can secrete some heterologous enzymes if it also contains genes which encode accessory factors such as the *pul*, *xcp* or *out* genes. It remains to be established whether or not a *pul*-like secretion apparatus is involved in the natural producers of these enzymes.

Like other extracellular proteins, the phosphatase precursor has a typical signal peptide which is necessary for its translocation across the cytoplasmic membrane. The presence of a leader peptide suggests that *Lysobacter* spp. possess Sec-like translocatory machinery and may secrete proteins in a two-step fashion (Wickner, *et al.*, 1991). The three examples of *L. enzymogenes* extracellular protein signal sequences, although differing slightly in size, have features common to all signal peptides. They have the positively charged N-terminal region, which consists of 1 to 4 arginine residues, followed by a core of hydrophobic or neutral residues, and the peptidase cleavage site which consists of small amino acids. Amino acid homology searches did not identify any sequences resembling the haemolysin C-terminal targeting signals. Thus, this would effectively eliminate the possibility that the phosphatase is secreted in a one-step process through both the cytoplasmic and outer membranes via a Hly-like secretion mechanism (Nicaud, *et al.*, 1986).

Possible functions of N-terminal propeptides have come mainly from work with proteases such as subtilisin E of *B. subtilis* (Ohta, *et al.*, 1991), proteases A and B of *S. griseus* (Henderson, *et al.*, 1987) and the α-lytic protease of *L. enzymogenes* (Silen, *et al.*, 1989). In addition to the signal sequence, these proteases are synthesized with a 100- to 200-residue N-terminal extension immediately preceding the mature protein (Nakahama, *et al.*, 1986; Henderson, *et al.*, 1987; Epstein and Wensink, 1988; Delepelaire and Wandersman, 1989). The N-terminal propeptides have been shown to be important in the folding and activation of the proteases and they belong to an interesting family called the co-translational chaperones (Ellis and van der Vies, 1991). The significance of this covalent association is that the chaperone does not have to diffuse through the medium to search for the protein that it is responsible for folding and activating. Furthermore, the N-terminal propeptide can exert its chaperone function when supplied exogenously to inactive mature enzyme (Silen and Agard, 1989). Precursors of the α-lytic protease have been observed to continuously fold and refold while the mature forms do not. It has been demonstrated that the propeptide is required to overcome a kinetic block in the folding pathway by lowering the free energy of the transition state rather than by preventing the formation of random non-productive intermediates. The active enzyme is believed to exist in a metastable state which is not necessarily the most thermodynamically stable (Baker, *et al.*, 1992; Creighton, 1992). In another study, a small deletion in the propeptide region of *B. cereus* neutral protease resulted in a delay in the detection of the active enzyme

(Wetmore, *et al.*, 1992). Propeptide-mediated folding may be a common feature in the processing and secretion of proteases. Thus, a possible role of the N-terminal propeptide of the phosphatase is that it may also act to prevent premature activation of the enzyme until translocation is complete and to aid in the proper folding of the protein. There is no apparent sequence homology between the propeptides of the phosphatase and the proteases since no protease sequences were selected by the homology searches. Therefore, any similarities between them would have to be functional.

A role for the C-terminal extensions of aqualysin I, protease I and the secreted phosphatase have not been determined, but they may be somehow involved in the secretion process. There are examples of proteases which contain C-terminal extensions, but not N-terminal propeptides in their precursor structure. The precursors of *Neiserria gonorrhoeae* IgA protease (Pohlner, *et al.*, 1987) and *S. marcescens* protease (Yanagida, *et al.*, 1986) contain large, hydrophobic C-terminal domains of greater than 400 residues. The C-terminal peptide of the IgA protease seems to be involved in the transport of the protease through the outer membrane by forming a pore. The *S. marcescens* protease may also be secreted using this mechanism. In contrast, the C-terminal domains of the secreted phosphatase, *Achromobacter* protease I and aqualysin I are less than half the size and relatively hydrophilic compared to those of the *Neiserria* and *Serratia* proteases, so it is therefore unlikely that they are able form a secretory pore. Although I can only speculate as to what the functions of the N- and C-terminal domains of phosphatase precursor may be, the *phoA* nucleotide sequence provides information which might be useful for further studies in order to determine their importance and possible roles in the folding, activation and secretion of the enzyme.

Little attention has been given to the purplish color of the *Lysobacter* phosphatase, which is apparent especially when the purified enzyme is highly concentrated. Amino acid homology analysis of the predicted amino acid sequence has determined that the secreted phosphatase appears to be related to a group of 34-40 kDa mammalian metalloenzymes known as Type 5, iron-containing, tartrate-resistant, purple acid phosphatases. This relationship is interesting because of the great evolutionary distance between bacteria and mammals, and furthermore, no other prokaryotic homologs have yet been discovered. Nevertheless, the similarity of the secreted phosphatase to the mammalian acid phosphatases in size, color and amino acid sequence through the proposed iron-binding/active site region suggested that the *Lysobacter* phosphatase may also be an iron protein. Indeed, atomic absorption analysis showed that a significant amount of iron was present in the purified protein sample. The absence of inhibition by iron chelators suggested either that the iron atom is inaccessible to the chelators or very tightly held by the enzyme.

The mammalian acid phosphatases are found in a variety of tissues including bovine spleen (Davis, *et al.*, 1981), porcine uterus (Schlosnagle, *et al.*, 1974), rat spleen and bone (Hara, *et al.*, 1984; Kato, *et al.*, 1986), human spleen, bone, placenta and macrophage (Ketcham, *et al.*, 1985; Hayman, *et al.*, 1989; Ketcham, *et al.*, 1989; Lord, *et al.*, 1990). In general, they have similar physical, immunological and functional properties. Magnetic and spectroscopic studies of the bovine spleen and uteroferrin isozymes have determined that there are two iron atoms associated with each enzyme (Gaber, *et al.*, 1979; Davis, *et al.*, 1981;Antanaitis and Aisen, 1982; Lauffer, *et al.*, 1983; Averill, *et al.*, 1987) whereas there appears to be only one for the secreted phosphatase of *L. enzymogenes*. Tyrosine and histidine residues have been implicated in the coordination of the iron atom (Davis and Averill, 1982; Lauffer, *et al.*, 1983). Bovine spleen acid phosphatase and uteroferrin are highly homologous and have ten conserved histidine and tyrosine residues which made it difficult to determine which residues were responsible for coordinating the iron atoms. The sequence "**LVGHDHNYQRY**", present in the C-terminal half of the *Lysobacter* secreted phosphatase and the mammalian acid phosphatases, is probably involved in the iron-binding/active site. The bold lettering indicates exact matches in the sequences. In the mammalian acid phosphatases, there is a similar sequence in the N-terminal half of the polypeptide "YLAGNHDHLGNVSAQIAY" (Ketcham, *et al*., 1989). The underlined residues common to both sequences may play a role in iron-coordination. The histidine and tyrosine residues are proposed to be directly involved with iron coordination (Vincent and Averill, 1990). The aspartate residue could accept a proton during the catalytic reaction. The upstream iron-binding sequence located in the N-terminal domain of the mammalian enzymes does not exist in the *Lysobacter* phosphatase. Therefore, it seems that both sequences are required to produce a diiron centre in the mammalian phosphatases and it is possible that the *Lysobacter* phosphatase can coordinate one iron atom with the one sequence. It is unlikely that the secreted phosphatase exists as a dimer that coordinates two iron atoms since purified phosphatase elutes from a Sephadex column at a point characteristic of a 25-30 kDa protein.

It is interesting to note that the α-lytic protease also has similarities with analogous enzymes from mammalian sources. The active site sequence, "GDSGG", occurs in the both the *Lysobacter* protease and mammalian serine proteases. Typical bacterial serine proteases have "GTSMA" sequence in the active site (Olson, *et al*., 1970; McLachlan and Shotton, 1971). There appears to be a distant evolutionary link between the active sites of some phosphatases and proteases of *Lysobacter* spp. and mammals.

Once the gene was cloned and characterized, the next step was to express the cloned gene in *E. coli*. Since it was uncertain whether the phosphatase precursor would be processed and activated properly to allow the determination of enzyme activity, it was necessary to have an alternative method to detect the presence of the expressed protein. Antiserum was raised against the native enzyme, but had low titres, about 1:500, perhaps due to the poor antigenicity of the phosphatase. Typical antibody titres for *Pseudomonas aeruginosa* pilin protein, a large molecular weight oligomer, are consistently over $1{:}10^6$ (K. Volpel, personal communication). However, the anti-AP obtained was able to inhibit the activity of the secreted phosphatase and distinguish it from the cell-associated phosphatase, *E. coli* alkaline phosphatase and the secreted phosphatase from *L. enzymogenes* ATCC

29488. During immunoblot experiments, it was found that sensitivity of detection was reduced by 100-fold or more in the presence of β-mercaptoethanol and prolonged heating. There are four cysteine residues in the mature enzyme. It is possible that the reduction of the two potential disulfide bonds would adversely affect the ability of anti-AP to recognize the phosphatase. Once the native conformation was altered by the denaturation step, the epitope was not recognized by anti-AP. This suggests that the antibody recognizes a discontinuous or conformational epitope, which is produced when relatively distant regions of the protein are brought together (Klein, 1990) and stabilized by hydrogen or disulfide bonds. The immunodetection experiments could possibly be improved using antibody produced by immunization with different adjuvants and/or heat denatured enzyme.

For the expression of the phosphatase, the pUC118-derived recombinant plasmid, called pES10, was examined since similar experiments with pUC118 containing the *Lysobacter* β-lactamase gene appeared to be successful in *E. coli* (Boras, *et al.*, 1993). Bands on the immunoblot corresponding to the secreted phosphatase, and, possibly, phosphatase precursors were detected in the shock fluids and Sephadex pools. The phosphatase signal sequence is apparently recognized by the *E. coli* Sec translocation system. At this stage, it is difficult to determine which of the intermediates correspond to the mature protein with N- or C-terminal extensions since they are about the same size, 42 692 Da and 40 678 Da, respectively. Furthermore, the apparent molecular weights were probably affected by the absence of β-mercaptoethanol in the samples. This would complicate molecular weight estimates since the protein may not be completely denatured and migrate accordingly. The fact that *phoA* was expressed was supported by the observation that the phosphatase from *E. coli* MV1193/pES10 shock fluid exhibited anti-AP-sensitive and EDTA-resistant activity. However, the yield of the phosphatase from *E. coli* MV1193/pES10 was very low. Compared to production by *L. enzymogenes*, only about 3% of phosphatase was found in the periplasm and the culture supernatants. This may be due to differences in codon usage and inefficiencies in transcription initiation, translation initiation and secretion. The phosphatase in the culture supernatant of *E. coli* MV1193/pES10 was probably due to leakage from the periplasm or a non-retentive outer membrane, but not due to secretion, since the β-lactamase was also found in the culture supernatant.

During the calibration of the Sephadex column, the secreted phosphatase activity eluted at a point centered around fraction 69 (Sephadex pool VI). As expected, the 28 891 Da TEM β-lactamase (Sutcliffe, 1978), which is encoded by pUC118, also eluted in pool VI. However, most of the phosphatase produced by *E. coli* MV1193/pES10 was found in pool V and did not co-elute with the β-lactamase. Thus, the phosphatase produced by *E. coli* eluted from the Sephadex column as a higher molecular weight species. This would suggest that the recombinant phosphatase may be an incompletely processed form of the enzyme. Alternatively, the phosphatase may have an altered Stoke's radius (Scopes, 1986) which would cause the protein to exhibit a different elution profile. The 30 kDa phosphatase and higher molecular weight phosphatase-like species were apparently recognized by anti-AP during immunoblotting experiments. During or after gel filtration, the intermediate form of the phosphatase seemed to be converted to the 30 kDa species. As shown in Results, the 3- to 6-fold activation of the enzyme was only observed when the shock fluid was passed through the column, but not after dialysis against the buffer used for gel filtration. Therefore, the activation is probably not due to the removal of a low molecular weight inhibitor such as phosphate. A large molecular weight inhibitor which can only be separated by gel filtration may be responsible. It is conceivable that the >11 kDa N- and/or C-terminal extensions may inhibit the phosphatase until it is completely secreted.

This project has been successful in the characterization and expression of the gene for the secreted phosphatase of *L. enzymogenes*. However, many questions still remain, especially those concerning the precise mechanism of enzyme activation, the roles that the N-terminal and C-terminal extensions may play in the processing and secretion of the phosphatase and the nature of the iron-coordination/active site. Before continuing with more extensive expression experiments, the efficiency of phosphatase production must be improved. One way this can be accomplished is to optimize translation initiation by converting the second codon from AAC to AAA or AAU, both of which are supposed to be at least three times more efficient in translation initiation (Looman, *et al.*, 1987). The conversion to AAA also replaces Asn-2 with Lys-2, which may cause the signal peptide to associate more strongly with the inner face of the cytoplasmic membrane in preparation for translocation. The use of stronger promotors may also be necessary to generate more mRNA to participate in translation. Furthermore, attempts to improve expression using a dicistronically organized vector system may permit over-production of the phosphatase (Ito and Kurosawa, 1992).

Expression of the recombinant phosphatase gene using T7 vectors and tac vectors has largely been unsuccessful so far . No enzyme activity was ever observed. *In vivo*-labelling experiments and immunoblots did not identify any expressed protein. However, the shock fluids from these cultures were neither concentrated nor applied to Sephadex columns. It is not certain whether any post-gel filtration activity would have been detected. Furthermore, the decreased sensitivity of immunoblotting caused by reducing agents was not known at the time. In retrospect, I would suggest that shock fluids should be concentrated and partially purified by gel filtration to maximize the amount of enzyme activity that can be recovered.

Once expression is improved significantly, perhaps in a different host organism such as *Pseudomonas* sp., the roles of the N- and C-terminal propeptides may be examined by experiments based on those done with the α-lytic protease (Silen and Agard, 1989; Silen, *et al.*, 1989; Baker, *et al.*, 1992). By engineering selected mutations within *phoA*, mutant phosphatase deleted for either or both extensions can be prepared in order to determine if the phosphatase can be produced, activated or secreted. Subsequently, the effect of exogenously supplied N- and/or C-terminal propeptide can be investigated. The structures of the N- and C-terminal domains can be altered by point mutations or small deletions to determine which regions in particular are important for the function of the propeptides. Furthermore, amino acid substitutions in the active site region can be made to determine which residues are important for the activity of the enzyme. It would also be of interest to compare the crystal structure of the *Lysobacter* phosphatase with the mammalian acid phosphatases in order to compare the 3-D structure around the iron-coordination/active site. Unfortunately, the amount of enzyme required for X-ray crystallography is not easily obtained. It may be possible, however, to examine the degree of similarity by determining whether the anti-AP antibody can cross-react with the mammalian phosphatases in immunoblot experiments.

More general investigations may include the development of a host-vector system for *Lysobacter* spp. much like those for *Streptomyces* spp. (Hopwood, *et al.*, 1985) or *Thermus* spp. (Touhara, *et al.*, 1991) and the isolation of mutants that are pleiotropically defective in protein secretion to determine whether a general secretion pathway similar to the *pul* or *xcp* systems exists in *Lysobacter*. Clearly, there are many aspects of protein secretion in *L. enzymogenes* that can be explored, not only with the phosphatase, but with the other secreted enzymes produced by *L. enzymogenes*.

CHAPTER 5

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